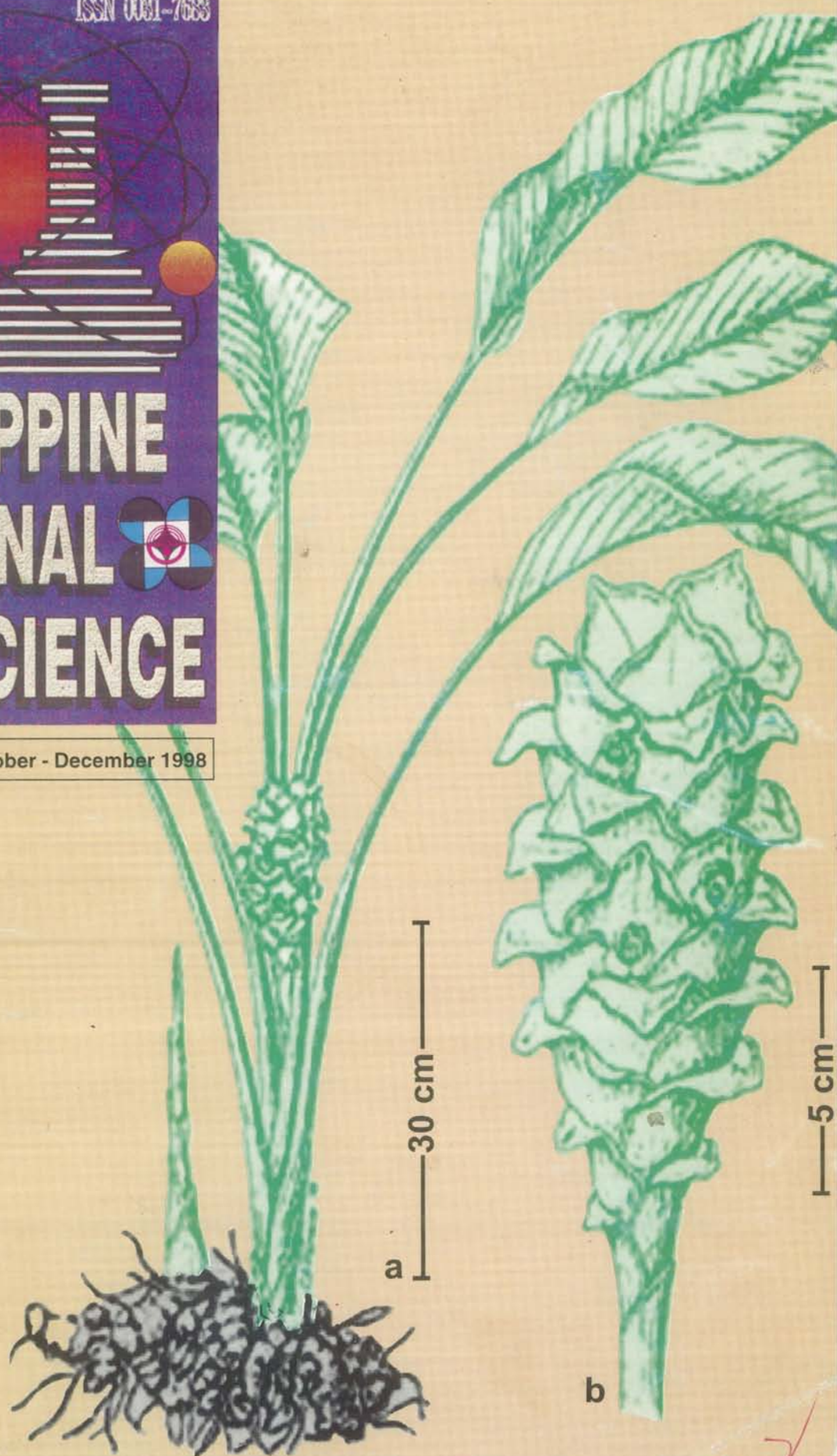


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*The cover, taken from the 1st manuscript (Figure 1) published in this issue, is a **Curcuma longa** L.*

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Isolation and Spectroscopic Studies of Curcumin from Philippine *Curcuma longa* L.

ROSALINDA C. TORRES, TERESITA S. BONIFACIO,
CELIA L. HERRERA and EDUARDO A. LANTO

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ABSTRACT

Curcumin, the yellow coloring matter was isolated from the rhizomes of Philippine Curcuma longa L. (turmeric) by Soxhlet extraction with toluene followed by concentration and slow crystallization. The isolated curcumin was then subjected to chromatographic and spectroscopic studies with the Merck curcumin standard. The Infra red and UV-Vis spectra of both compounds were found to be almost identical indicating a high purity of the isolate. The % yield obtained was 2-3%.

INTRODUCTION

Curcuma longa L. is a finger or long turmeric that belongs to the family Zingiberaceae. The plant (Figure 1) (Co, 1989) is widely distributed in the Philippines. The rhizomes are commonly sold in the markets of Manila, and are used as a condiment, as an ingredient of curry powder and as food coloring. Turmeric is one of the best known of material dyes, being used for dyeing silk, wool and cotton. The rhizomes are also used medicinally as stimulant, tonic, carminative, emmenagogue and astringent. These are prepared as powder, paste, ointment, oil and lotion. Turmeric paste is a popular application for sprain and bruises, while the ointment is used for neuralgia and rheumatism (de Padua, 1978; Quisumbing, 1978)

The rhizomes of *Curcuma longa* L. contains 3-4% curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepta-diene-3, 5-diene, demethoxycurcumin and bisdemethoxycurcumin (Figure 2). These compounds belong to the group of diarylheptanoids and comprise the so-called curcuminoids, the yellow coloring constituent of *C. longa* and *C. xanthorrhiza*. Curcumin is a non-steam



Figure 1. The plant, *Curcuma longa* L.
a. Habit of the whole plant;
b. Inflorescence

volatile diferuloyl- and dicinnamoyl-methane compound (Tonnesen, et al. 1983; Wagner, et al. 1984).

Curcumin, the natural coloring matter of turmeric possesses the indicator properties first noted for turmeric in 1815 by H. A. Vogel. Both the Eastman and the Matheson, Coleman and Bell Catalogs of organic chemicals definitely identify by formulation or C.I. No; the curcumin offered as C.I. No. 75300 and give pH ranges of 7.4 to 8.6 and 7.8 to 9.2 for the yellow to brownish orange color shift. Curcumin colors wool, silk and cotton greenish yellow; after alum mordanting, orange yellow; chrome, brown; iron, brownish black; tin, orange red (Lilli, 1969).

The therapeutic action of curcumin was likewise reported. It was shown to have anti-inflammatory and antifatulent activity. Moreover, it was recommended for gastric and duodenal ulcer dyspepsia and stomachache (ASOMPS, 1992).

With the demand for natural colors and a never-ending search for biologically-active natural products, it is therefore of interest to develop a method for the isolation of highly purified curcumin from locally available curcuma species. The use of spectroscopic methods of infra red and ultraviolet-visible light were employed in the determination of the purity of the compound isolated.

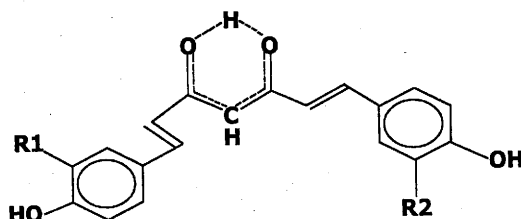


Figure 2. The main colored substances of *Curcuma longa* L.

$R_1 = R_2 = \text{OCH}_3$

Curcumin

$R_1 = \text{OCH}_3, R_2 = \text{H}$

Demethoxycurcumin

$R_1 = R_2 = \text{H}$

Bisdemethoxycurcumin

EXPERIMENTAL

Materials

Fresh rhizomes of *Curcuma longa* L. were washed thoroughly and peeled off thinly. These were then cut into cubes and weighed. Weighed samples were placed in a cheesecloth bag and subjected to hydrosteam distillation for the extraction of essential oil. A cleverger tube apparatus was used for the collection and separation of the oil. Distillation was continued until no more oily drops were collected. The oil collected was treated with anhydrous sodium sulfate and filtered. The oil-free turmeric rhizomes were then removed from the cheesecloth bag and air-dried for several days. The dried rhizomes were powdered, in preparation for the isolation of curcumin.

Isolation of Curcumin

Dry turmeric powder was extracted with toluene in a Soxhlet apparatus for 5 hours. The toluene extract was concentrated under vacuum and allowed to crystallize at room temperature. A yellow-orange crystalline powder was obtained which was then subjected to infra-red and UV-V is spectroscopic studies and thin layer chromatography.

Thin-layer Chromatography (TLC) of Isolated Curcumin

Preliminary test by TLC was carried out on silica gel 60G with CH_2Cl_2 -MeOH (98+2) as solvent system. Spots were viewed under ultraviolet light at 254 nm and 366 nm, respectively. A Merck curcumin standard of 99% purity was used for qualitative comparison with the isolate.

Spectroscopic Studies of Isolated Curcumin

The isolated curcumin was subjected to ultraviolet-visible (UV-Vis) spectroscopy at a concentration of 200 ppm with ethanol using Shimadzu UV-VIS-NIR Model UV-3100. Infra red (IR) spectroscopy was likewise conducted using Shimadzu infra red spectrophotometer in a KBr pellet.

RESULTS AND DISCUSSION

Hydrosteam distillation of fresh turmeric rhizomes prior to curcumin extraction yielded 0.3 % (ml/g) of the essential oil. The oil-free and ground turmeric rhizomes were then extracted with toluene in a Soxhlet apparatus for 5 hours. Concentration of the extracts under vacuum and allowing the concentrate to crystallize for some time produced 2-3 % curcumin.

Preliminary testing of the isolated curcumin by thin-layer chromatography (TLC) was then conducted on a silica gel 60G with CH_2Cl_2 :MeOH (98:2) as solvent system. Results (Figure 3) revealed the presence of three (3) yellow spots as seen by the naked eye. The same spots, however, were fluorescent greenish yellow when viewed at 366 nm of UV light. No spot was visible at 254 nm. Merck curcumin standard produced the same three (3) yellow spots with almost the same R_f values. The results strongly indicate the purity of the isolated curcumin.

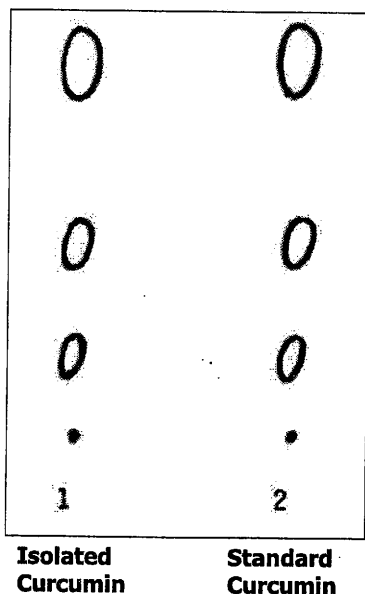


Figure 3. Chromatogram of the isolated curcumin and a Merck standard as carried out on silica gel 60G with CH_2Cl_2 :MeOH (98:2) solvent system and viewed under light at 366 nm.

To further verify the purity of the isolate, spectroscopic studies of IR and UV-vis were employed. UV-vis spectra are shown in Figures 4, 5 and 6; while the IR spectra are presented in Figure 7 and 8. From the absorption curve (Figure 4), the isolated curcumin had a maximum absorption at 424.0 nm which indicated a chromophore and a two conjugated system at 199.5 nm. An almost identical chromophore and a two conjugated system at 199.5 nm. An almost identical absorption curve (Figure 5) was seen in the Merck curcumin standard suggesting a much higher purity of the isolate. Figure 6, which showed a curcumin standard superimposed with the isolated curcumin, further verified the purity of the standard.

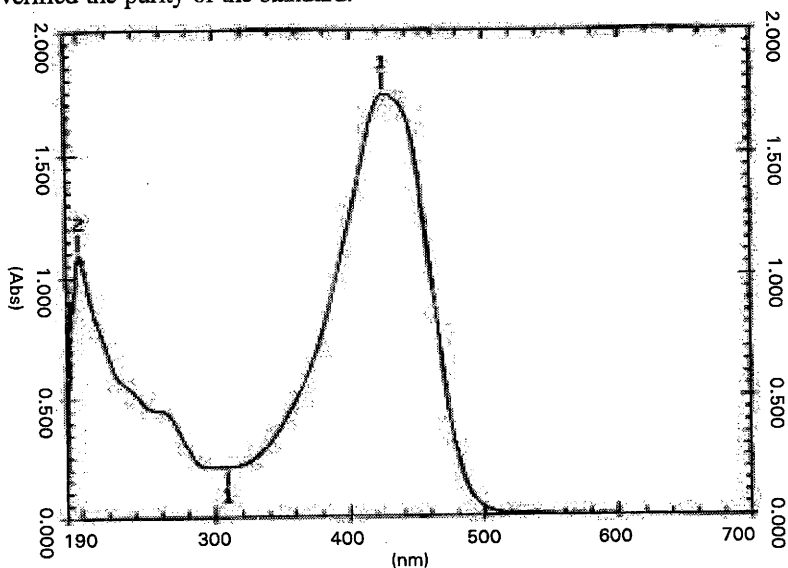


Figure 4. UV-Vis spectrum of the isolated curcumin.

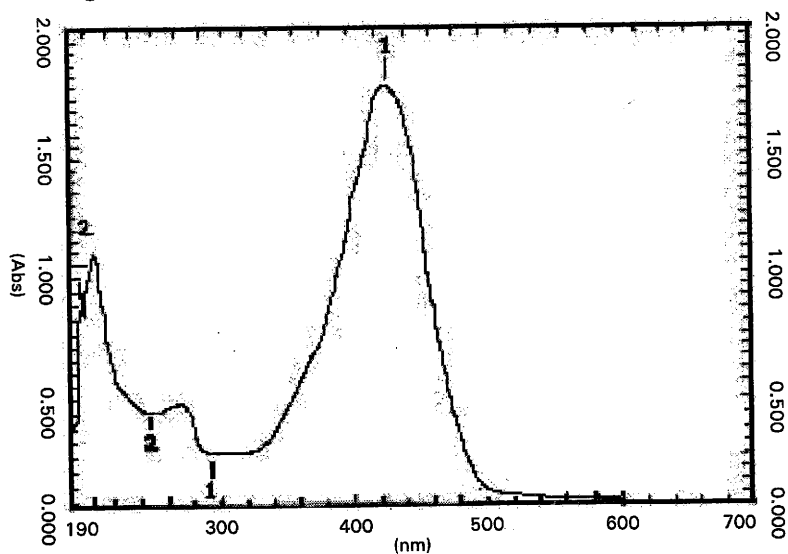


Figure 5. UV-Vis spectrum of the Merck curcumin standard.

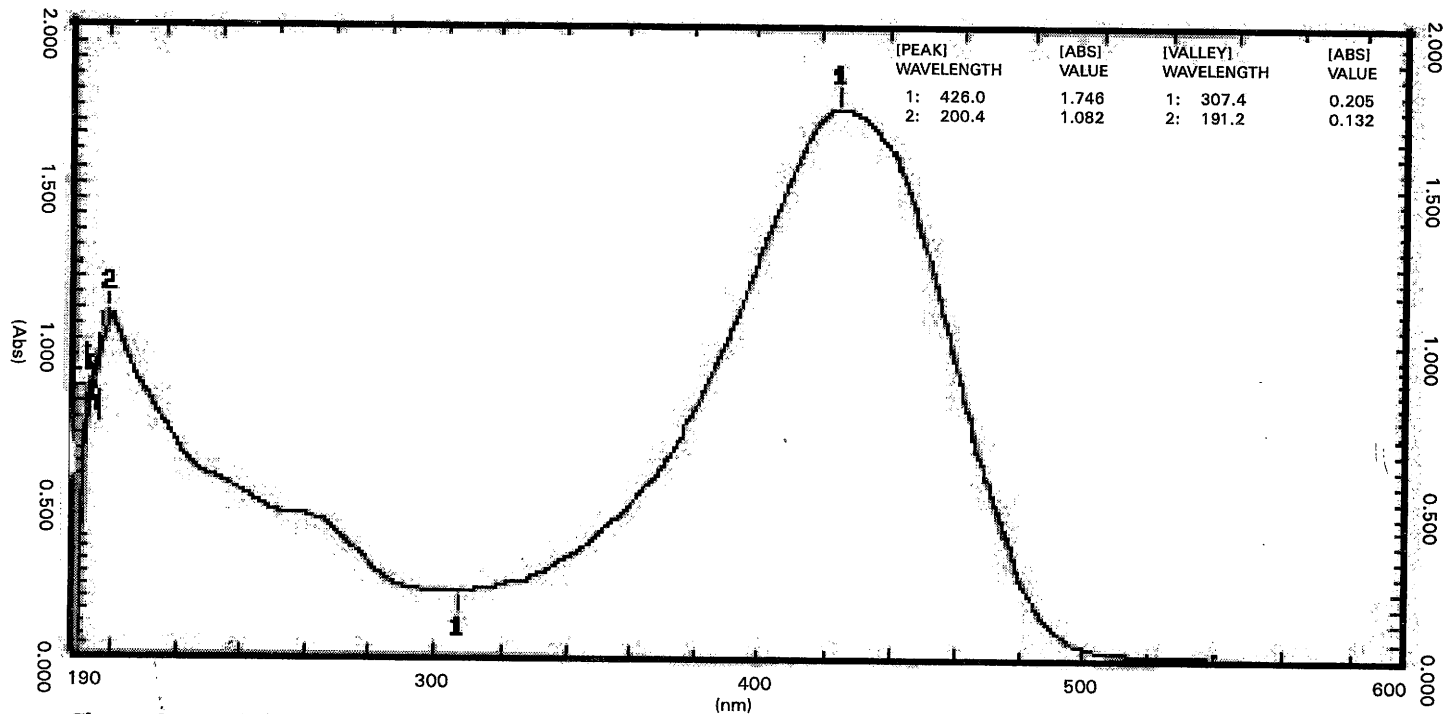


Figure 6. UV-Vis spectrum of a curcumin standard as superimposed with the isolated curcumin.

Infra-red spectrum data of the isolated curcumin (Figure 7) showed the following data; -COCHCOH- band at $1400\text{-}1300\text{ cm}^{-1}$, double bonds of -C=C- at $1667\text{ - }1667\text{ cm}^{-1}$ and an aromatic ring at $1600\text{ - }1513\text{ cm}^{-1}$. The sharper peaks indicated that the compound had a very high purity. Almost the same IR spectrum was observed in Merck curcumin standard as shown in Figure 8.

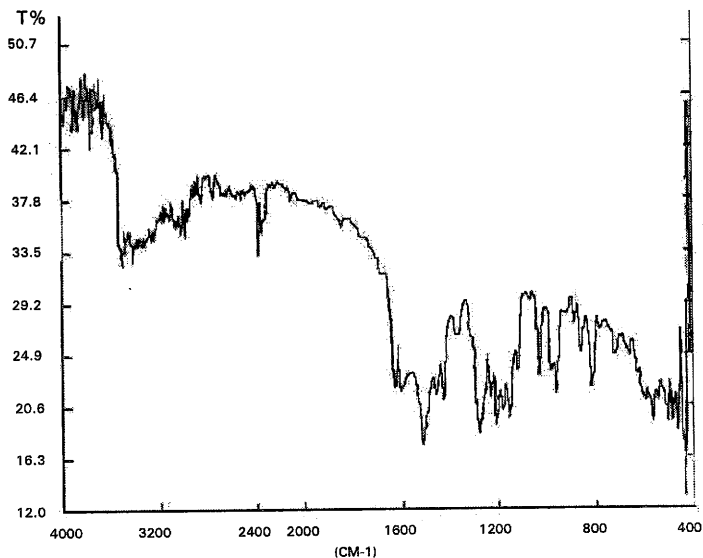


Figure 7. Infra-red spectrum of the isolated curcumin in KBr pellet.

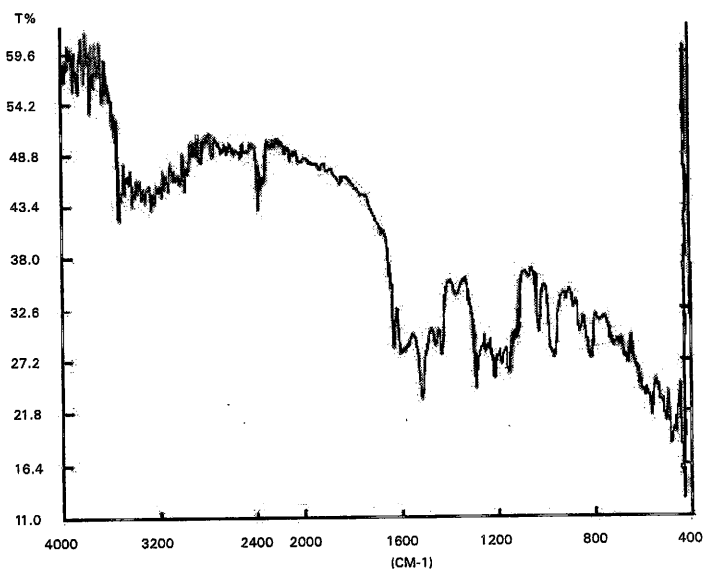


Figure 8. Infra-red spectrum of the Merck curcumin standard in KBr pellet.

ACKNOWLEDGMENT

The authors gratefully acknowledge the late Mrs. Josefina P. Manalo and Mrs. Felicisima D. Uñalivia for the technical assistance and valuable suggestions for the completion of the project, and to Ms. Ma. Cecelyn S. Cruz for typing the manuscript.

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Philippine Vegetable Drugs in Common Use: Their Chemical Constants

Part I

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ABSTRACT

This paper provides a ready reference for the chemical constants/properties of 28 commonly used vegetable drugs in the Philippines as a guide for laymen, industry and other entrepreneurs/users for their identification and quality/purity determination.

Proximate chemical components of these drugs namely: moisture, total ash, acid-insoluble ash, crude fiber, volatile oil and the volatile ether, non-volatile ether, hexane, dilute alcohol, alcohol and water soluble extractives respectively were determined. The methods specified in the United States Pharmacopoeia 20th revision were followed.

INTRODUCTION

The renewed interest in the use of vegetable drugs nowadays could be due to the increasing prices of imported drugs and emergence of many side-effects of synthetic medicinals. It is noteworthy that there are quite a number of publications dealing on medicinal plants such as Medicinal Plants of the Philippines (Quisumbing, 1950), Useful Plants of the Philippines (Brown, 1958), The Guidebook on the Proper Use of Medicinal Plants (Maramba et al, 1962), four volumes on Handbook on Medicinal Plants (de Padua et al, 1977, 1978, 1981, 1983) and the Philippine National Formulary (NSTA, 1982). These books and publications contain reported information on the medicinal uses, preparations, constituents isolated from plants with their scientific, local, English and Spanish names together with the family to which each plant belongs. Since a number of these plants/plant parts are potential sources of vegetable drugs, the need for a ready reference of their chemical components or properties becomes imperative to serve as a guide for laymen, industry and other users in the identification and quality/purity determination of each drug. The determination of chemical constants/properties using Pharmacopoeial methods of analysis is an initial step in establishing the official standards of all vegetable drugs sold in Metro Manila markets and other parts of the country.

The objective of the study is to determine the total ash, acid-insoluble ash, moisture, crude fiber, volatile oil and extractives soluble in alcohol, dilute alcohol, solvent hexane, water and ethyl ether to serve as database for the common vegetable drugs which are frequently used for medicinal purposes.

MATERIALS AND METHODS

Most of the twenty eight (28) vegetable drugs (Table I) used in the study were bought from herbal vendors in Quiapo while others were collected within the gardens of Metro Manila. Except for "bawang" and "sibuyas bombay" which were used as fresh samples, the materials freed from all dust and dirt were cut into small pieces, air-dried at room temperature or dried at 50 - 60 °C in a fabricated drying cabinet and reduced to a coarse powder in a Wiley Mill.

The Methods of Chemical Analysis for Vegetable Drugs (USP XX, 1980) were used in the determination of the chemical constants/properties of the twenty-eight (28) commonly used vegetable drugs. Acid-insoluble ash method was used to determine foreign inorganic impurities. To determine moisture content, the gravimetric method was used while the azeotropic method (toluene distillation) was used for volatile oil-containing drugs (Fig. 1). The amount of volatile oil was determined by distillation with

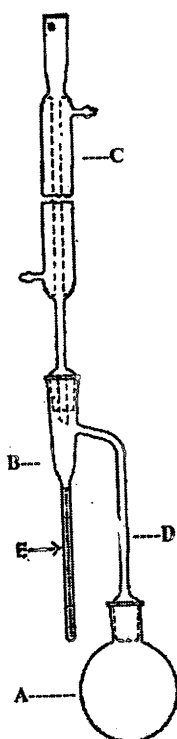


Figure 1. Toluene Moisture Apparatus.

A - One (1) liter glass flask for sample and toluene

B - Trap

C - Condenser

D - Connecting tube

E - Receiving tube for condensed moisture and toluene

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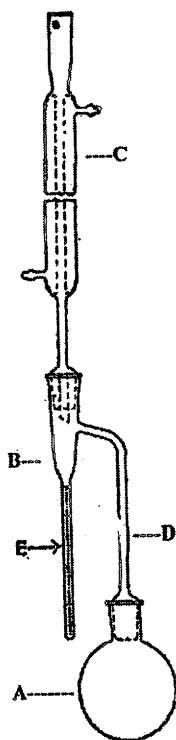


Figure 1. Toluene Moisture Apparatus.

- A - One (1) litter glass flask for sample and toluene**
- B - Trap**
- C - Condenser**
- D - Connecting tube**
- E - Receiving tube for condensed moisture and toluene**

water using the clavenger apparatus for collection and separation of the volatile oil (Fig. 2). The extractives containing the active constituents were determined by continuous extraction of the drug for a specified period of time with solvent such as alcohol, solvent hexane and diethyl ether using Soxhlet extraction apparatus (Fig. 3) and by maceration of the drug with dilute alcohol and water for 24 hours.

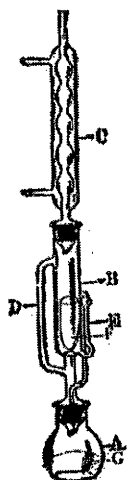
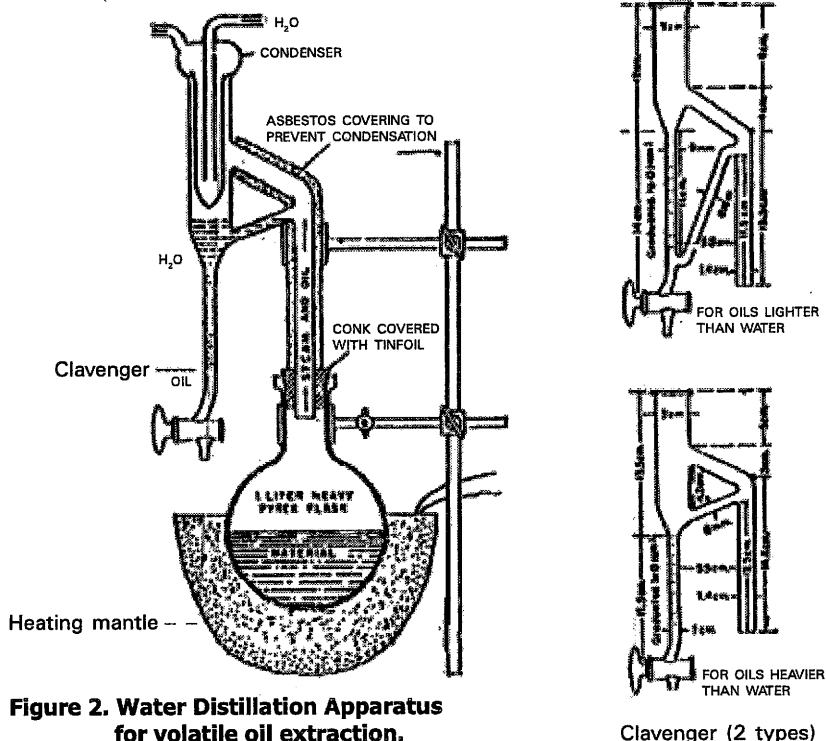


Figure 3. Soxhlet Extraction Apparatus for determination of Alcohol Soluble, Hexane Soluble and Ether Soluble Extractives.

- A - Tared flask
- B - Extraction tube
- C - Condenser
- D - Side tube
- E - Siphon tube
- F - Extraction timble
- G - Solvent

RESULTS AND DISCUSSION

Based on their various medicinal actions reported in literature as shown in Table I, these twenty eight (28) vegetable drugs were selected for the first part of the study. For convenience in identifying the drug, the scientific name, common name, English name if any and the family to which it belongs is also included in the same table.

The purity of vegetable drugs depends on the absence of foreign matters, organic or inorganic. According to pharmacopoeial standards, maximum of 2 percent acid-insoluble ash representing the inorganic matter is permissible in vegetable drugs (USP XX, 1975). Table 2 shows vegetable drugs containing more than 2 percent acid-insoluble ash, namely "damong maria," "tanglad" and "ulasimang-bato" therefore not meeting the USP specification. Considering that the materials are properly cleaned and freed from dirt and dust before reducing them to powder, their high acid-insoluble ash contents are inherent to the plant parts used as drug.

Moisture is normally present in all dried drugs to the extent of 5 to 10 percent (Claus et al, 1967). Table 2 shows moisture or water contents of the drugs determined generally by gravimetric method and by toluene distillation method for those drugs containing volatile oil and other volatile components. For proper storage of vegetable drugs, moisture content must not exceed 10 percent. Any excess in moisture makes the drug susceptible to the promotion of mold and bacterial growth which subsequently cause their deterioration and spoilage. Some of the crude drugs analyzed contain more than 10% moisture (10.42 - 10.9%) but they were analyzed as soon as they were air-dried so that there was no possibility of mold growth on the samples tested. For drugs that are to be stored for long periods of time, moisture content must be less than 10%.

The quality of vegetable drug refers to the intrinsic value of the drug, that is the amount of medicinal principle or active constituents contained in the drug. These constituents are determined by assay. Table 3 shows the results of assay for extractives of drugs. The amount of extractives, that is the amount of medicinal principle or active constituents present in the drug such as glycosides, saponins, tannins, alkaloids, sterols, hormones and other soluble compounds obtained by continuous extraction in solvents such as alcohol, solvent hexane, water and diethyl ether are presented. The amount of extractive that a drug yields to a given solvent is often an approximate measure of the amount of a certain constituent or group of related constituents that the drug contains (Jenkins et al, 1949). By proximate assay, the amount of inert matter such as total ash and crude fiber

Table 1. Common, English/Spanish and scientific names, families, parts used and medicinal actions.

Common Name	English/Spanish	Scientific Name	Family	Parts Used	Medicinal Actions
Abutra	Barberry	<i>Arcangelisia flava</i> Linn.	Menispermaceae	wood	Germicide, stomachic, febrifuge, purgative, tonic, expectorant
Achuete	Annatto	<i>Bixa orellana</i> Linn.	Bixaceae	leaves	Febrifuge, purgative, diuretic
Adelfa	Oleander	<i>Nerium indicum</i> Mill.	Apocynaceae	leaves bark	Abortifacient, sternatory, diuretic
Alibagbang		<i>Bauhinia malabarica</i> Roxb.	Leguminosae	leaves	Febrifuge
Ampalaya	Balsam pear	<i>Momordica charantia</i> Linn.	Cucurbitaceae	tender shoots/young leaves	Vulnerary, stomachic, antipyretic, purgative
Avocado	Avocado/Allitor Pear	<i>Persea americana</i> Mill.	Lauraceae	leaves	Pectoral, stomachic
Banaba		<i>Lagerstroemia speciosa</i> (Linn.) Pers.	Lythraceae	leaves bark	Antidiabetic, diuretic, stimulant, febrifuge, antidiarrhetic
Bawang	Garlic, Ajos	<i>Allium sativum</i> Linn.	Liliaceae	bulbs	Diaphoretic, diuretic, expectorant, stimulant, anti-hypertensive
Bayabas	Guava	<i>Psidium guajava</i> Linn.	Myrtaceae	leaves & bark	Astringent, vulnerary, antidiarrhetic, anti-spasmodic, antiseptic
Chichirica	Pink periwinkle	<i>Catharanthus roseus</i> (Linn.) Don	Apocynaceae	leaves	Antidiabetic, emmenagogue, antidyspeptic
Damong Maria	Santa Maria, Mugwort	<i>Artemisia vulgaris</i> Linn.	Compositae	leaves	Expectorant, Carmi-native, tonic, antiseptic
Eucalyptus	Eucalyptus	<i>Eucalyptus globulus</i> Labil	Myrtaceae	leaves	Stimulant, antiseptic
Fistula	Golden shower	<i>Cassia fistula</i> Linn.	Leguminosae	fruit	Purgative, laxative, cathartic, antirheumatic
Gumamela	Hibiscus	<i>Hibiscus rosasinensis</i> Linn.	Malvaceae	leaves	Emollient, febrifuge, aperient, anodyne
Kalabasa	Squash	<i>Cucurbita maxima</i> Duchesne	Cucurbitaceae	seeds	Antihelminthic, vermifuge, diuretic
Kalachuche	Temple flower	<i>Plumiera acuminata</i> Air.	Apocynaceae	bark	Rubefacient, anti-herpetic, counter irritant, febrifuge
Kamias	Cucumber Tree	<i>Averrhoa bilimbi</i> Linn.	Oxalidaceae	leaves	Germicide, anti-rheumatic
Kinchai	Celery	<i>Apium graveolens</i> Linn.	Umbelliferae	whole plant	Carminative, stimulant, tonic, diuretic, emmenagogue
Makabuhay		<i>Tinospora rumphii</i> Boerl.	Menispermaceae	stems	Diuretic, stimulant, demulcent
Makahiya	Bashful mimosa	<i>Mimosa pudica</i> Linne	Leguminosae	stems	Alterant, antiasthmatic
Pandan mabango	Fragrant Srewpine	<i>Pandanus odoratissimus</i> Linn.	Pandanaceae	leaves	Cardiotonic, purgative
Pakwan	Watermelon, sandia	<i>Citrulus vulgaris</i> Schrad.	Cucurbitaceae	seeds	Diuretic, vermifuge, demulcent, pectoral, antihypertensive
Paminta	Black Pepper	<i>Piper nigrum</i> Linn.	Piperaceae	seeds	Rubefacient, stimulant, febrifuge, stomachic
Papaya	Papaw	<i>Carica papaya</i> Linnae	Caricaceae	leaves	Antirheumatic, anti- asthmatic
Sabila	Aloe	<i>Aloe vera</i> Linn.	Liliaceae	leaves	Purgative, antibaldness, emollient, vulnerary
Sibuyas Bombay	Onion/Cebollas	<i>Allium cepa</i> Linn.	Liliaceae	bulbs	Antiseptic, diuretic, emmenagogue
Tanglad	Lemon Grass	<i>Andropogon citratus</i> DC	Graminae	leaves	Stomachic, tonic, diuretic, refrigerant
Ulasiman bato		<i>Peperomia pellucida</i> (Linn.) HBK	Piperaceae	whole plant	antirheumatic, diuretic

contained in each drug were determined as shown in Table 2. It was observed that some of the drugs have a high percentage of crude fiber ranging from 21 to 39.4 percent and total ash ranging from 10.7 to 21.74 percent. By physical assay, using the water distillation method, the volatile oil, the active constituent of volatile oil-containing plants was determined and results are also shown in Table 2.

Table 2. Total ash, acid insoluble ash, crude fiber, moisture and volatile oil content.

Common Name/ Parts Used	Total Ash %	Acid Insoluble Ash %	Crude Fiber %	Moisture %	Volatile Oil %
Abutra stems	7.10	0.59	36.42	4.33	negative
Achuete leaves	3.85	0.72	13.84	10.42	1.76
Adelfa leaves,	6.48	0.38	14.20	8.67	negative
bark	7.29	0.08	18.30	6.07	negative
Alibagbang leaves	9.03	1.12	21.03	7.69	negative
Ampalaya tender leaves	14.45	1.95	6.63	10.80	negative
Avocado leaves	5.04	0.93	13.75	10.75	0.01
Banaba bark	6.02	0.48	39.40	8.65	negative
Bawang bulbs (fresh)	1.45	0.03	1.60	39.92	0.14
Bayabas leaves	3.28	0.72	10.62	9.83	0.86
Chichirica leaves	11.34	0.44	7.44	10	0.03
Damong Maria leaves	11.60	4.07	15.51	8.98	0.36
Eucalyptus leaves	7.03	0.27	8.15	8.76	1.31
Fistula pulp	5.30	2.58	11.50	9.91	negative
Gumamela leaves	10.70	0.01	9.03	10.71	negative
Kalabasa seeds	4.82	0.026	0.12	8.33	negative
Kalachuchi bark	4.70	0.35	31.30	6.98	negative
Kamias leaves	9.46	0.12	13.40	7.87	negative
Kinchai whole plant	13.75	0.55	11.50	10.52	0.04
Makabuhay stems	7.87	0.45	23.08	6.50	negative
Makahiya whole plant	4.69	0.04	34.22	10.79	negative
Pandan mabango leaves	9.86	0.94	27.39	9.98	traces
Pakwan seeds	3.86	0.05	5.52	3.51	negative
Paminta seeds	3.97	0.05	7.50	10.90	0.40
Papaya leaves	9.53	0.012	14.13	10.80	negative
Sabila leaves	25.6	0.30	6.2	8.44	0.54
Sibuyas Bombay bulbs (fresh)	0.34	0.027	0.35	76.42	0.01
Tanglad leaves	7.02	2.44	6.19	9.55	0.99
Ulasiman bato whole plant	21.74	3.20	8.39	7.26	negative

Table 3. Soluble extractives.

Common Name/ Parts Used	Alcohol %	Dilute- Alcohol %	Solvent Hexane%	Water %	Non-Volatile Ether %	Volatile Ether %
Abutra stems	3.86	12.02	0.62	5.62	1.06	0.13
Achuete leaves	8.34	25.62	4.42	15.92	4.94	0.80
Adelfa leaves,	12.70	11.27	3.70	10.34	10.20	0.32
bark	3.65	9.56	3.32	8.30	4.45	0.32
Alibagbang leaves	9.38	10.76	2.63	14.18	4.62	0.15
Ampalaya tender leaves	17.00	26.00	0.85	22.00	3.62	0.85
Avocado leaves	22.80	11.82	3.40	9.77	5.90	0.31
Banaba bark		11.89	1.08	11.34	0.81	0.07
Bawang bulbs (fresh)	22.26	28.38	0.91	26.12	0.36	1.08
Bayabas leaves	25.35	22.50	0.31	21.77	0.19	0.70
Chichirica leaves	28.46	30.24	5.31	28.56	7.96	0.36
Damong Maria leaves	16.29	11.66	4.10	14.46	5.17	0.38
Eucalyptus leaves	27.80	28.62	6.20	17.53	12.41	0.80
Fistula pulp	35.83	79.53	0.95	81.13	2.96	0.57
Gumamela leaves	12.05	11.67	3.79	17.50	4.40	0.49
Kalabasa seeds	45.95	2.55	38.11	4.38	52.75	1.74
Kalachuchi bark	30.34	12.24	1.60	10.38	3.45	0.65
Kamias leaves	19.55	25.05	3.83	17.92	4.21	0.21
Kinchai whole plant	28.16	32.62	7.07	31.76	2.96	0.57
Makabuhay stems	18.44	13.43	2.32	12.80	3.95	0.40
Makahiya whole plant	7.14	3.96	1.07	2.59	1.86	0.06
Pandan mabango leaves	12.67	6.95	3.00	12.00	3.28	0.59
Pakwan seeds	38.77	3.33	49.36	4.20	48.95	0.81
Paminta seeds	3.88	12.82	4.70	7.50	0.55	0.62
Papaya leaves	28.05	24.90	8.90	25.37	8.40	0.70
Sabila leaves	2.60	16.72	2.20	25.50	2.15	0.26
Sibuyas Bombay bulbs (fresh)	15.92	7.22	0.65	5.22	0.42	0.24
Tanglad leaves	8.23	14.85	1.89	13.68	2.80	0.12
Ulasiman bato whole plant	16.93	24.73	4.68	22.89	4.6	1.00

SUMMARY AND CONCLUSION

Moisture contents of eight (8) out of twenty eight (28) of the dried crude drugs tested were not monitored to give the necessary limit of less than 10% for proper storage (>10% but <11%) but this has no adverse effect on the results of the analysis since the drugs were analyzed soon after they have been dried. Two (2) of the samples namely *Allium sativum* L. and *Allium cepa* L. were analyzed as fresh samples because it takes time to air-dry them without being decomposed.

Total ash indicates the amount of inorganic components of the drug. Results showed that of the twenty eight (28) plant materials analyzed, five (5) produced more than 10% total ash and two (2) had more than 20% total ash namely "ulasiman-bato" and "sabila". This indicates that these two (2) plants contain plenty of inorganic salts. Acid-insoluble ash of four (4) plants were over the limit of two percent for purity but since the plants were cleaned or washed before drying, the ash contents are inherent to the plant materials analyzed.

The amount of extractive that a drug yields to a given solvent represent the amount of active constituents or mixture of related constituents contained in the drug. Therefore the alcohol and water soluble extractive yielded by the drugs can be used as basis for their quality standard. For volatile oil-containing drugs the yield of volatile oil based on moisture free drug can be used as basis for their quality.

The crude fiber content of the drugs as indicated is of considerable importance in the examination of certain drugs particularly of spices, since the commonly used adulterants consist of waste or refuse material derived from the drugs or spices themselves or from food products. Therefore the increase or decrease of crude fiber indicates the presence of adulterants.

The tests and assays undertaken are official requirements for vegetable and animal drugs either imported or exported. The data obtained in the study can serve as an initial step in establishing the official standards of all vegetable drugs grown throughout the country.

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Photocatalytic Reduction of Nickel (II)

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ABSTRACT

The photocatalytic reaction of Ni(II) on zinc oxide powder was carried out. Photocatalytic degradation of Ni(II) was observed spectrophotometrically. The effect of varying some parameters, such as the pH, concentration of Ni(II), amount and particle size of the semiconductor, and light intensity on the rate of photocatalytic reaction was observed. A tentative mechanism for the reduction of Ni(II) is proposed.

INTRODUCTION

Nature nowadays suffering from a serious problem of environmental pollution. Industrial effluents, a major of pollutants is found to contain a number of metal ions which are toxic to aquatic life. There are various techniques to remove these pollutants; each one has its own advantages and limitations. Photocatalytic treatment of such effluents may prove to be more beneficial than the other routine techniques and therefore the present work was undertaken.

Organic contaminants in water can be completely mineralised by irradiation in the presence of semiconductor TiO_2 (1-6). Photocatalysed degradation of methyl vinyl ketone (an industrial pollutant) in aqueous solution has been carried out by Munner et al (7). Tanaka et al (8) reported photocatalytic degradation of chloral hydrate in aqueous semiconductor suspension. Recently, Soni (9) proposed a mechanism to explain the photocatalytic degradation of methylene chloride, chloroform, and carbon tetrachloride.

Tennakone et al (10) reported TiO_2 catalysed oxidative photodegradation of mercurochrome; the same research group used anthracene colloid for photoextraction of silver from aqueous solution (11). Wang and Zhuang (12) have investigated photocatalytic reduction of the pollutant Hg(II) ion on doped WO_3 suspension. Recently, photocatalytic reduction and immobilization of Cr(VI) at TiO_2 in aqueous basic media was reported by Lin et al (13). Photoreduction of Fe(III) in marine mineral aerosol solution has been reported by Zhu et al (14). Inel and Ertek (15) observed photocatalytic deposition of Bi(III) ions on titania powder. Heterogeneous photocatalytic oxidation on Mn(II) over TiO_2 has been reported by Lozano et al (16), while photocatalytic reduction of the environmental pollutant Cr(IV) on CdS powder was observed by Wang et al (17). Photocatalytic reduction of Hg(II) has been observed by Wang et al (17).

Photocatalytic reduction of Hg(II) and Cu(II) ions (18,19) in illuminated aqueous suspension of ZnO powder has also been reported. Mollers et al (20) discussed photocatalytic deposition of noble metals.

Nickel comes from hydrogenation in the oil industry and paint factories. The present study describes the photocatalytic reduction of Ni(II) in water over ZnO powder.

EXPERIMENTAL DETAILS

Nickel sulphate (Merck) and zinc oxide (E. Merck) were used in present investigation. Stock solution of Nickel sulphate was prepared in doubly distilled water. The photocatalytic reduction of Nickel sulphate was observed by taking 50.0 ml solution (4.0×10^{-2} M) in a 100 ml beaker and 0.5g of zinc oxide was added to it. Irradiation was carried out keeping the whole assembly exposed to a tungsten lamp (light intensity 38.0 mWcm^{-2}). The intensity of light at various distances from the lamp was measured with the help of a solarimeter (Surya Mapi Model CEL 201). A water filter was used to cut out thermal radiations. The pH of the solution was measured with a digital pH meter (Systronics Model 324). The desired pH of the solution was adjusted by the addition of previously standardized sulphuric acid and sodium hydroxide solution. The necessary condition for the correct measurement of the optical density is that the solution must be free from semiconductor particles and impurity; a centrifuge (REMI - 1258) was used to remove these species. The amount of ZnO, temperature and light intensity was kept 0.5g, 303 K and 38.0 mWcm^{-2} respectively throughout the experiment.

The progress of the photocatalytic reaction was observed by taking optical density at regular time intervals using spectrophotometer (Jasco UV 7800).

RESULT AND DISCUSSION

The photocatalytic degradation of Nickel sulphate was observed at $\lambda_{\text{max}} = 400 \text{ nm}$; the result of a typical run are given in Table 1 and represented graphically in Figure 1. It has been observed that a plot of log (optical density) v/s time is linear, which indicates that this photocatalytic reduction follows first order kinetics. The rate constant of this reaction was calculated from the relation,

$$k = 2.303 \times \text{slope}$$

Table 1. A typical run.[Nickel sulphate] = 4.0×10^{-2} M

pH=5.0

 $\lambda_{\text{max}}=400$ nm

Time (min)	Optical Density (O. D.)	1 + log O. D.
0.0	0.134	0.1271
20.0	0.132	0.1238
40.0	0.131	0.1173
60.0	0.130	0.1139
80.0	0.128	0.1072
100.0	0.127	0.1038
120.0	0.126	0.1004
140.0	0.124	0.0934
160.0	0.123	0.0892
180.0	0.122	0.0863
200.0	0.121	0.0828
220.0	0.120	0.0792
240.0	0.119	0.0755
260.0	0.117	0.0682
280.0	0.116	0.0645
300.0	0.115	0.0607

 $k=8.36 \times 10^{-6} \text{ sec}^{-1}$

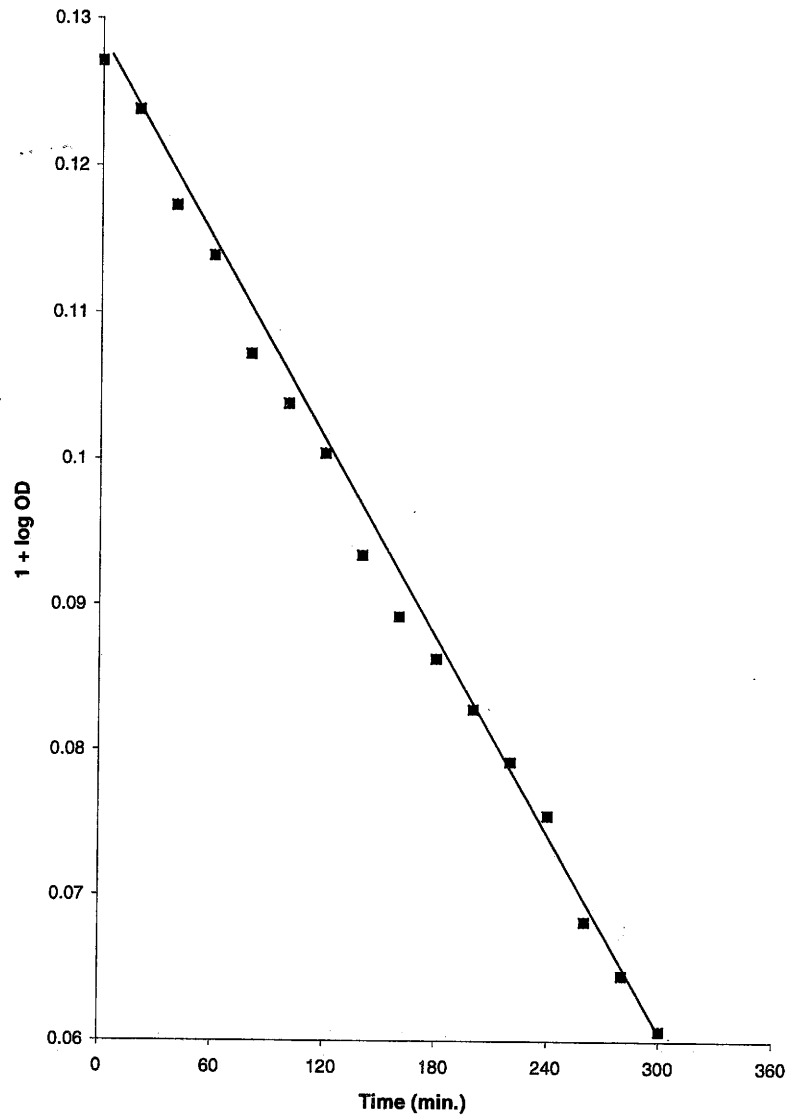


Figure 1. A typical run.

Effect of pH

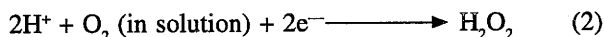
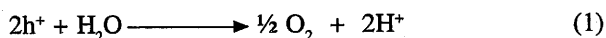
The effect of pH on the rate of reduction of Ni(II) was investigated in the pH range 2.5 - 6.5; because above pH = 6.5 precipitation of nickel hydroxide was observed. The reaction rate was found to increase as the pH was increased and an optimum value was obtained for pH = 5.0. The result are reported in Table 2.

Table 2. Effect of pH.

[Nickel sulphate] = 4.0×10^{-2} M

pH	$k \times 10^6$ (sec ⁻¹)
2.0	5.15
2.5	5.60
3.0	6.01
3.5	6.98
4.0	7.36
4.5	8.00
5.0	8.36
5.5	7.51
6.0	5.25
6.5	4.60

This pH dependence can be explained on the basis that hole can generate H^+ ions in solution from water whereas these protons are utilized by dissolved oxygen (in solution).



These two reactions counterbalance each other to a particular extent. It is now known that processes at the semiconductor-electrolytic interface depend on the surface properties of the semiconductor (21). The pH, where the net charge on the surface of the semiconductor is zero, is called the point of zero charge (pzc) (21). The value of point of zero charge can be lowered if anions are adsorbed on the surface of the semiconductor (22). The reverse may be true if cations are adsorbed. The available data indicate that the photocatalytic degradation of nickel sulphate proceeds smoothly when the surface is slightly positively charged; the reaction rate was maximum at pH = 5.0. Also for pH > 5.0, equation (2) starts

dominating the equation (1), so that there will be a further decrease in the amount of H^+ ions and hence, the decrease in rate of photocatalytic degradation of $Ni(II)$ species.

Effect of Nickel Sulphate Concentration

In the present study, the concentration of $Ni(II)$ varies between ranges 1.00 and $7.14 \times 10^{-2} M$; the results are given in Table 3.

Table 3. Effect of nickel sulphate concentration.

pH=5.0

[Nickel sulphate] $\times 10^2 M$	$k \times 10^6 (sec^{-1})$
1.00	3.12
1.31	3.45
1.74	4.01
2.00	4.60
2.32	5.18
2.65	5.86
2.90	6.49
3.26	7.22
3.55	7.84
4.00	8.36
4.41	7.48
4.64	6.51
5.03	5.20
5.48	4.31
6.00	3.52
6.51	2.88
7.14	2.10

As the concentration of Nickel sulphate was increased, there was an increase in the rate of the reaction. This may be explained on the basis that as the number of substrate molecules increases, there will be a corresponding increase in the rate of the reaction. However if the concentration of $Ni(II)$ was increased above $4.0 \times 10^{-2} M$, the coloured solution will absorb the major portion of the incident light and it may not permit the desired light intensity to fall on the semiconductor powder. Thus a decrease in the rate of photocatalytic degradation was observed.

Effect of the amount of Photocatalyst

The effect of amount of photocatalyst on the rate of photocatalytic removal of Ni(II) was also observed by using different amounts of semiconductor. The result are reported in Table 4.

Table 4. Effect of amount of photocatalyst.

[Nickel sulphate] = $4.0 \times 10^{-2} \text{M}$

pH=5.0

ZnO (g)	$k \times 10^6 (\text{sec}^{-1})$
0.10	1.61
0.15	2.18
0.20	2.91
0.25	4.32
0.30	5.48
0.35	6.39
0.40	7.20
0.45	7.82
0.50	8.36
0.55	8.37
0.60	8.36
0.65	8.36
0.70	8.34
0.75	8.36

It has been observed that k increases with an increase in the amount of zinc oxide. This increase in the rate of reduction may be attributed to an increase in the exposed surface area of the semiconductor. However after a certain limit (0.55g) has been reached, if the amount of zinc oxide was increased further, there was no increase in the exposed surface area of the photocatalyst. This is the saturation point, above which an increase in the amount of semiconductor has no appreciable effect on the rate of photocatalytic degradation of Ni(II).

It was further confirmed by using vessels of different capacities; the saturation point was shifted to higher values for larger vessels and showed a downward shift for smaller vessels. It was further supported by the observation that this point shifts to higher side, when the solution was stirred.

Effect of Light Intensity

An effect of light intensity on the photocatalytic removal of Ni(II) was also observed. The results are summarized in Table 5.

Table 5. Effect of light intensity.

[Nickel sulphate] = $4.0 \times 10^{-2}M$

pH=5.0

Intensity of Light (mWcm ⁻²)	k x 10 ⁶ (sec ⁻¹)
12.0	0.02
15.0	2.61
18.0	3.49
21.0	4.03
24.0	4.90
27.0	5.72
30.0	6.30
34.0	7.29
38.0	8.36
40.0	8.91
44.0	9.55

The rate of photocatalytic reaction increases with increase in the light intensity. It may be attributed to the fact that with an increase in intensity of light, more electron-hole pairs will be generated; thus more electrons will be available for reducing the substrate and hence, the rate of photocatalytic reaction will be increased.

Effect of Particle Size

To observe the effect of particle size of the photocatalyst on the photocatalytic reduction of Ni(II), semiconductors of different particle sizes were used. The result are reported in Table 6.

Table 6. Effect of particle size.

[Nickel sulphate] = $4.0 \times 10^{-2}M$

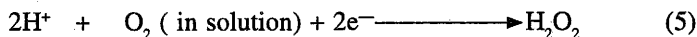
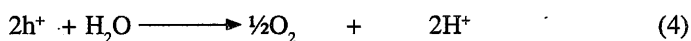
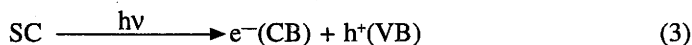
pH=5.0

Particle size (μm)	k x 10 ⁶ (sec ⁻¹)
0.8	8.36
1.6	5.16
2.4	4.01
3.2	3.22
4.0	2.46

It can be seen that, as the particle size of the semiconductor was increased, there was a corresponding decrease in the reaction rate. This may be due to the fact that, on increasing the particle size of the photocatalyst, the overall surface area of the semiconductor will decrease, thus resulting in a decrease in the rate of photocatalytic removal of Ni(II).

Mechanism

On the basis of the observed data, the following tentative mechanism is proposed for the photocatalytic degradation of Nickel Sulphate.



The semiconductor (SC) is excited on exposure to visible light, which will provide an electron (e^-) in the conduction band and a hole (h^+) in the valance band. This hole may be utilised by the water molecule to generate oxygen and H^+ ions. These H^+ ions and dissolved oxygen in solution can be reduced by two electrons to form hydrogen peroxide, which may slowly degrade. In the last step, Ni(II) may accept two electrons from the semiconductor and will reduce to its metallic state. This reduction of Ni(II) is clearly indicated by the lustrous grey deposit on the semiconductor zinc oxide.

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Cloning and Partial Sequence Analysis of Asterosaps from *Asterias amurens* (Starfish)

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ABSTRACT

Two complementary DNA clones 2.2 kb and 1.8 kb that encodes the sperm activating peptides (asterosaps) were isolated from an ovary cDNA library of *Asterias amurens* (Starfish). Partial DNA sequence predicted the peptide fragment made up of dibasic amino acid pair of Lys-Arg in both N and C termini. The 2.2 kb clone is composed of asterosaps 9-1, 13-2 and 13-1, the same isoforms isolated from egg jelly coat which have the ability to activate homologous spermatozoa and induce acrosome reaction in combination with an egg jelly glycoconjugate named ARIS. The DNA sequence of 1.8 kb clone predicted a structure composed of asterosaps 9-1 and 13-2 with two new isoforms that were not observed in the egg jelly coat. Deduced amino acid sequence of the two new isoforms: 4A and 4B predicted a peptide structure of Gly-Gln-Thr-Gln-Phe in the N-terminal region. Isoform 4A is similar to asterosap 13-2 but with substitution of alanine with valine in position 22 from the amino terminal. Isoform 4B has the same amino acid sequence with asterosap P3. Variation was observed at position 25 from the amino terminal in which phenylalanine was substituted by valine. Subcloning of 2.2 and 1.8 kb inserts in *Sma* 1 and *Stu* 1 generated a single DNA molecule. Deduced amino acid sequence predicted another two new isoform of asterosaps which were not found in egg jelly coat. Isoform 4C is similar to 13-1 but with substitution at position 25 from the amino terminal with valine while isoform 4D is similar to 13-2 with substitution also observed at position 25 from the amino terminal with valine. The various asterosaps found in egg jelly coat suggested in part, variable structure within a single copy of mRNA. The peptides may be processed by a protease.

INTRODUCTION

The eggs of *Asterias amurens* (starfish) contain a group of peptides capable of activating the homologous spermatozoa and inducing acrosome reaction (Nishigaki *et al* 1996). These sperm-activating peptides (SAPs)

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are one of the bioactive components of egg jelly coat in echinoderms (Garber, 1989). Thus these substances are involved in the process of fertilization (Hoshi, 1994). Twelve sperm-activating peptides (Figure 1) from the egg jelly coat were isolated from *Asterias amurensis* and eleven were structurally identified by sequence analysis and electro-spray ionization mass spectrometry (Nishigaki *et al* 1996). These are glutamine-rich tetratriacontapeptides with an intramolecular disulphide linkage between Cys⁸ and Cys³² much larger than the SAPs of sea urchin which have no significant sequence similarities to known proteins. Thus, they called these substances as asterosaps.

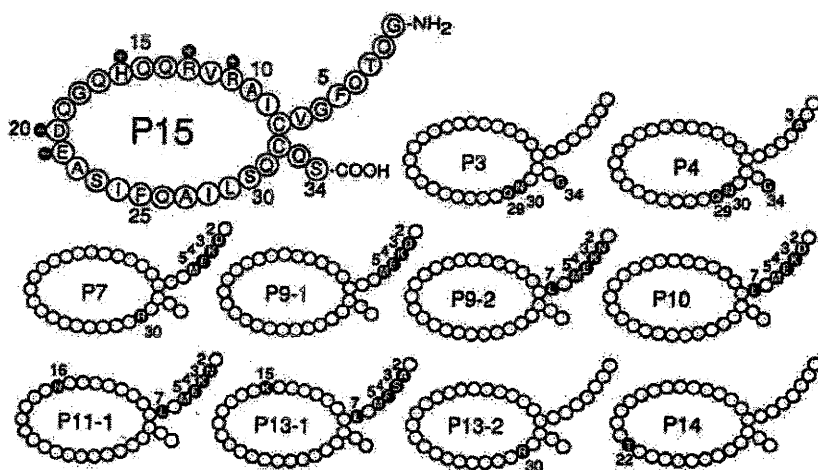


Figure 1. Structure of asterosaps isolated from egg jelly coat of *Asterias amurensis*. P 15 is shown as the representative. Others are shown as isoform peptides and the filled circles represent amino acid changes compared with P 15. The numbers indicate positions counted from the amino terminal. Ionized forms of basic and acidic residues are shown in P 15. (From Nishigaki, T. *et al* 1996, *Zygote* 3: 237 -245)

According to Ramarao *et al* (1990), multiple copies of egg peptides speract and its isoforms are encoded in a single mRNA. They were able to obtain a complementary DNA clone (2.3 kb) that encodes the egg peptide speract (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) isolated from an ovary cDNA library of the sea urchin *Strongylocentrotus purpuratus*.

The objective of this study is to identify other isoforms of asterosaps and determine the structure of precursor polypeptide. To achieve this goal, the experimental design is mainly involved in the cloning of asterosaps from the ovary of *Asterias amurensis*. The mRNA of asterosaps can be cloned as complementary DNA (cDNA). The constructed cDNA library will represent the information encoded in the mRNA of starfish ovary of which

individual processed segments of SAPs from the original genetic information can be isolated and examined. The amino acid sequence will be determined and the results will be compared with asterosaps isolated from the egg jelly coat.

MATERIALS AND METHODS

Construction of sap cDNA Library

Total RNA was prepared from immature ovary of *Asterias amurensis* (starfish) according to the method of Chomczynski and Sacchi (1987). The poly (A)⁺ fraction was isolated using an oligo-dT column matrix (Takara, Biomedicals, Japan) based on standard protocol (Sambrook et al, 1989). The cDNA library was constructed by means of the ZAP II cDNA Synthesis Kit (Stratagene, La Jolla, Calif.) following the manufacturer's instructions.

Screening of cDNA Library

The cDNA fragment was random-prime labelled with [³²P] - dCTP. Using this probe, screening was done as follows: pre-hybridization in 6 x SSC buffer, 20 mM KH₂PO₄, 0.4% (w/v) SDS, salmon sperm DNA (500 ug/mL) and 5 x Denhardt's reagent for 2 hours then incubated at 42°C and hybridized overnight in 6 x SSC buffer, 20 mM KH₂PO₄, 0.4% (w/v) SDS with salmon sperm DNA (500 ug/mL) at 65°C. This was then followed by washing in 6 x SSC at RT for 5 minutes and another in 2 x SSC containing 0.1% SDS using the same parameters and a final washing in 0.1 x SSC containing 0.1% SDS at 65°C for one hour.

The cDNA library was screened following the conditions as described above. About twenty NZY agar plates (150mm) containing 40,000 pfu/plate were transferred to filters and screened. The filters were exposed to X-OMAT XAR film (Eastman Kodak Co., Rochester, NY) at - 80°C overnight. The strongest "putative" clones were determined and a secondary screening was made in 10 NZY agar plate (100 mm) with 50~200 phage per plate following the same conditions as described above.

Single Clone Excision

The plaque where the cDNA positive clones were observed was cored and transferred to a sterile micro-centrifuge tube containing 50 uL of SM buffer and 20 uL CHCl₃. The phage stock solutions were stocked at 4°C. Excision was done in vivo using ex-Assist helper phage with SOLR strain following the protocol described in ZAP II cDNA Synthesis Kit (Stratagene, La Jolla, Calif.).

Plasmid Preparation/DNA Purification

This was done using "Promega" WizardTM Plus Minipreps DNA Purification System (Promega corporation, Madison, USA) following the instruction's manual.

Amplification of cDNA

This was carried out by polymerase chain reaction (PCR) using Q1 and Q2 as primers. Q1 has a nucleotide sequence of CA (A/G) CA (A/G) CA (C/T) CA (A/G) GGNCAAGA while Q2 is composed of CGNTA (T/G/A) (G/A) AN (A/T) (C/G) X GT (T/C) AC (A/G) GT. Amplification was done using 30 PCR cycles at 94 °C (1min), 46 °C (2 min) and 72 °C (1 min). The amplification products were then analyzed by electrophoresis.

DNA Sequencing

Size characterization of inserts was performed using the restriction enzymes Kpn I and Sac I, an internal site located within the adapter. Both 5' and 3' extremities of the two specific clones were sequenced from double-stranded cDNAs. DNA sequencing was performed using a DNA Sequencer Model 4000 (LI-COR, Aloka, Japan) with commercially available forward and reverse sequencing primers (LI-COR, INC. Japan). The cycle sequence was followed according to cycling conditions established by Aloka, Limited (Japan) using a PTC-100TM Programmable Thermal Controller (MJ Research Inc., Mass. USA). The nucleotide and amino acid sequence were analyzed using the GENETYX-MAC Software Development Co., LTD. suite of programs. These were then compared with known SAP sequence.

RESULTS

The mRNA of SAP isolated from the ovary of *Asterias amurensis* was subjected to polymerase chain reaction (PCR) using Q1 and Q2 primers and the products were analyzed in agarose gel electrophoresis. Q1 and Q2 primers were derived from glutamine since SAP structure showed a glutamine-rich peptide. The results are shown in Figure 2. Different DNA fragments were formed as observed in the appearance of several bands in lane 1. The DNA fragments were then transferred to nylon membranes and hybridized to one of the fragment using [32 P] - dCTP. After hybridization, the filters were exposed to X-OMAT XAR film and as observed in lane 2, the bands formed were similar to original sample (lane 1).

To determine the amount and size of total RNA in an ovary, RNA was separated from the oocytes and follicle cells then transferred to a nylon membrane and hybridized with labelled asterosap cDNA probe using [32 P] - d CTP. The result is shown in Figure 3. The faint band found in lane 1 corresponds to follicle RNA and lane 2 for oocytes RNA. This

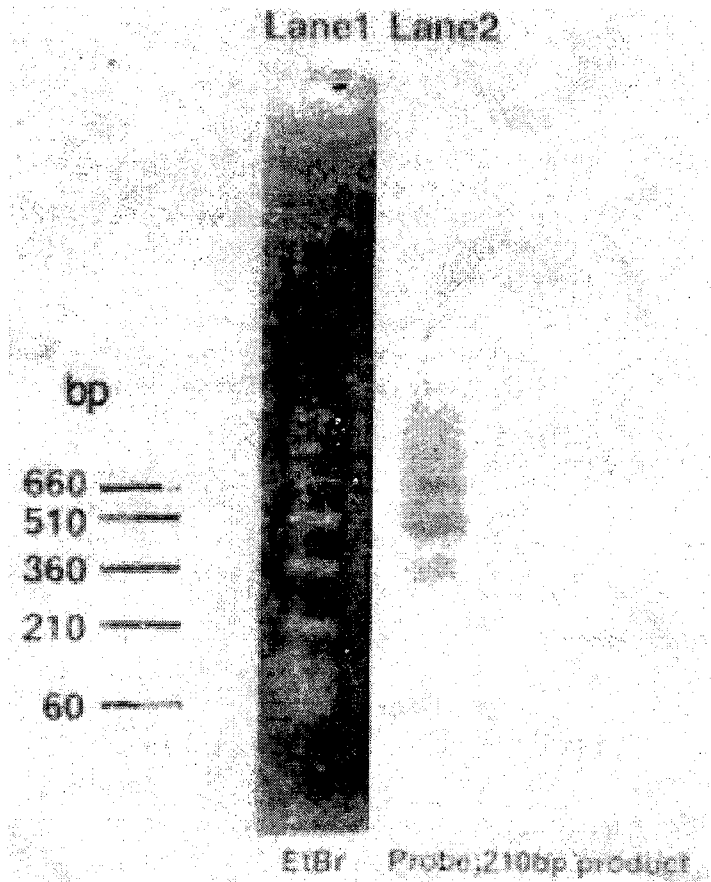


Figure 2. Resolution of SAP mRNA by agarose gel electrophoresis using polymerase chain reaction (PCR). Lane 1 is stained with ethidium bromide and lane 2 by hybridization probe.

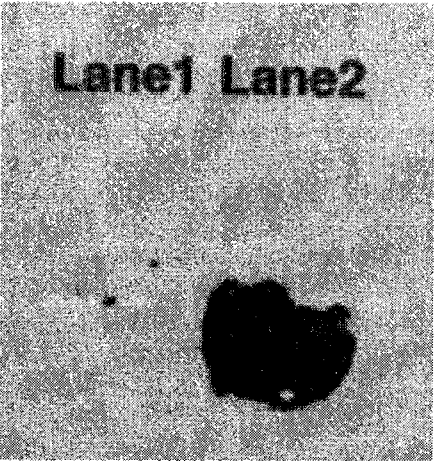


Figure 3. Northern blot of SAP mRNA from follicle and oocytes.

suggested that RNA is mostly found in oocytes of an ovary than in the follicle cells which supports our assumption that SAP can also be found in an ovary. The size is about 3.7 kb as estimated using the northern blot analysis.

About nine positive clones were selected from first screening. Further screening produced two cDNA clones. The cDNA clones, SAP 3 and SAP 4 were subjected to polyacrylamide electrophoresis to analyze the restriction fragment length polymorphism. This was carried out by the polymerase chain reaction using Q1 and Q2 primers. Figure 4 showed distinct bands suggesting different DNA fragments. The profile is similar to the profile of SAP mRNA found in Figure 2 (lane 1).

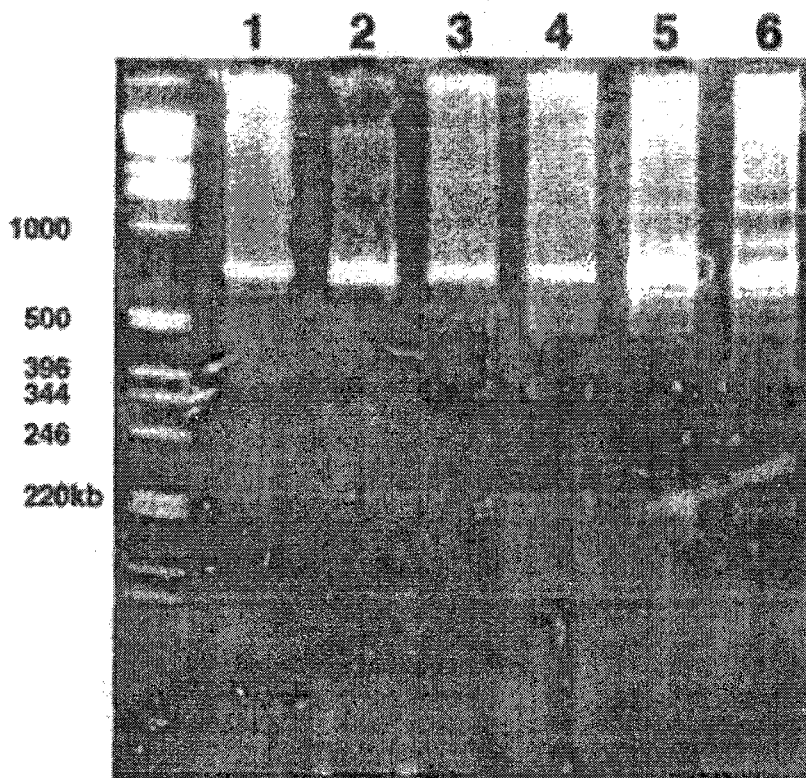


Figure 4. Resolution of DNA fragments from cDNA clones (single colony) by polyacrylamide gel electrophoresis using PCR.

Single-clone excision was performed from the above-mentioned cDNA clones. A plasmid was prepared and purified. This was again subjected to polyacrylamide electrophoresis using polymerase chain reaction using the same primers (Q1 & Q2). Faint bands were obtained at 210, 360, 660 bp for both asterosaps (data not shown).

The purified DNA of both SAPs was digested with Kpn 1 and Sac 1 restriction enzymes to determine the length of DNA insert using 1 kb DNA ladder as marker. This was then subjected to agarose gel electrophoresis. Figure 5 showed that SAP 3 has a vector size of 3.0 kb while the DNA insert is about 2.2 kb. SAP 4 has the same vector size with SAP 3 but a shorter DNA insert of about 1.8 kb.

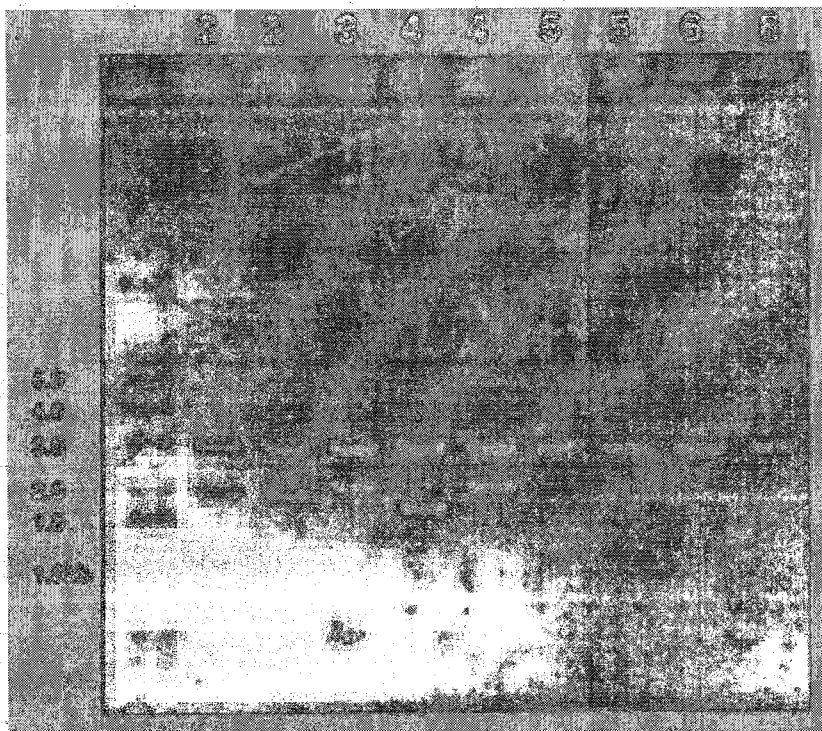


Figure 5. Estimation of the size of DNA fragments in an agarose gel.

Partial DNA sequence of SAP 3 & 4 is shown in Figures 6 & 7. Lys-Arg residues are shaded. Both precursors have an alternating units of asterosap and pro-peptide fragment.

The analysis of amino acid sequence of SAP 3 showed the presence of asterosap (Gly-Asp-Lys-Glu-Met-Gly-Val-Cys-Ile-Ala-Arg-Val-Arg-Gln-Gln-His-Gln-Gly-Gln-Asp-Glu-Ala-Ser-Ile-Phe-Gln-Ala-Ile-Leu-Ser-Gln-Cys-Gln-Ser) corresponding to asterosap 9-1 (Figure 1). Another asterosap (Gly-Gln-Thr-Gln-Phe-Gly-Val-Cys-Ile-Ala-Arg-Val-Arg-Gln-Gln-His-Gln-

**Partial nucleotide and amino acid sequences
of 2.2 kb insert (SAP 3)**

10	20	30	40	50	60
GATAAAGAGATGGGGGTATGTCGCTCGAGTAAGACAACAACATCAAGGTCAAGACGAA					
D K E M G V C I A R V R Q Q H Q G Q D E					
70	80	90	100	110	120
GCCITCACTTTCAAGCCATCTTGAGTCAATGTCAAGACGAGAAATACCAAAAGAT					
A S I F Q A I L S Q C Q S K R E I T K D					
130	140	150	160	170	180
CCTCCAGTGAAAGATGATGAAACAAAAGAGGTCAAACACAATTTGGTGTATGTCATCGCT					
P P S E D D E T K R G Q T Q F G V C I A					
190	200	210	220	230	240
CGAGTAAGACAACAACATCAAGGTCAAGACGAGCTTCATCTTTCAAGCCATATTGAGA					
R V R Q Q H Q G Q D E A S I F Q A I L R					
250	260	270	280	290	300
CAATGTCAAGACGAAACGAAATACCAAAGATCTCCCACTGAAGATGATGAAACAAA					
Q C Q S K R E I T K D P P S E D D E T K					
310	320	330	340	350	360
AGGGGTCAAACACAATTTGGTGTATGTCGCTCGAGTAAGACAACAACATCAAGGTCAA					
R G Q T Q F G V C I A R V R Q Q G Q G Q					
370	380	390	400	410	420
GACGAAGCCTCCATCTTTCAAGCCATCTTGAGACAATGTCAAGACGAAACGAGAAATCAGC					
D E A S I F Q A I L R Q C Q S K R E I S					
430	440	450	460	470	480
AAAGACCCCTCCCACTGAAGATGATGATGAAACCAAGAGAGGTGATAAAGAGATCGGTGTA					
K D P P S E D D E T K R G D K E M G V					
490	500	510	520	530	540
TGTATCGCTCGAGTAAGACAACAACATCAAGGTCAAGACGAAAGCCTCCATCTTCCAAGCC					
C I A R V R Q Q H Q G Q D E A S I F Q A					
550	560	570	580	590	600
ATCTTGAGCCCAATGTCAAGACGAAACGAGAGATGTCCTTTCCTTTAAGTGAACACAGCAA					
I L S Q C Q S K R E M S L P L S E T Q Q					
610	620	630	640	650	660
AACCAAGAGACGAAGACATCAAGAGAGGTGCTTCAGAGATGGGTTTATGTATCGCTCGA					
N Q E D E D I K R G A S E M G L C I A R					
670	680	690	700	710	720
GTAAGACAAAAACATCAAGGTCAAGACGAAGCCTCCATCTTCCAAGCGCATCTTGAGTCAA					
V R Q K H Q G Q D E A S I F Q A I L S Q					
730	740	750	760	770	780
TGTCAGACGAAACGAGAAATACCAAAGAGTCCTCCCGTGAGTGATGAACCAAAAGGGT					
C Q S K R E I T K E S S P * V M N Q K G					
790	800	810	820	830	840
CAAACACAATTTGGTGTATGATCGCTCGAGTAAGCACAAACATCAGGTCAAGCGCAGATC					
Q T Q F G V C I A R V S T T S G Q A S I					
850					
ATCGTACAAGCCAT					
I V Q A					

Figure 6. Partial nucleotide and amino acid sequences of 2.2 kb insert (SAP 3). The numbers located on top are the nucleotide positions. Dark shaded amino acids represent the astersaps and the pro-peptide fragments are shaded with gray. The dibasic pair lys-arg of the pro-peptide fragment is shaded with light gray.

**Partial nucleotide and amino acid sequences
of 1.8 kb insert (SAP 4)**

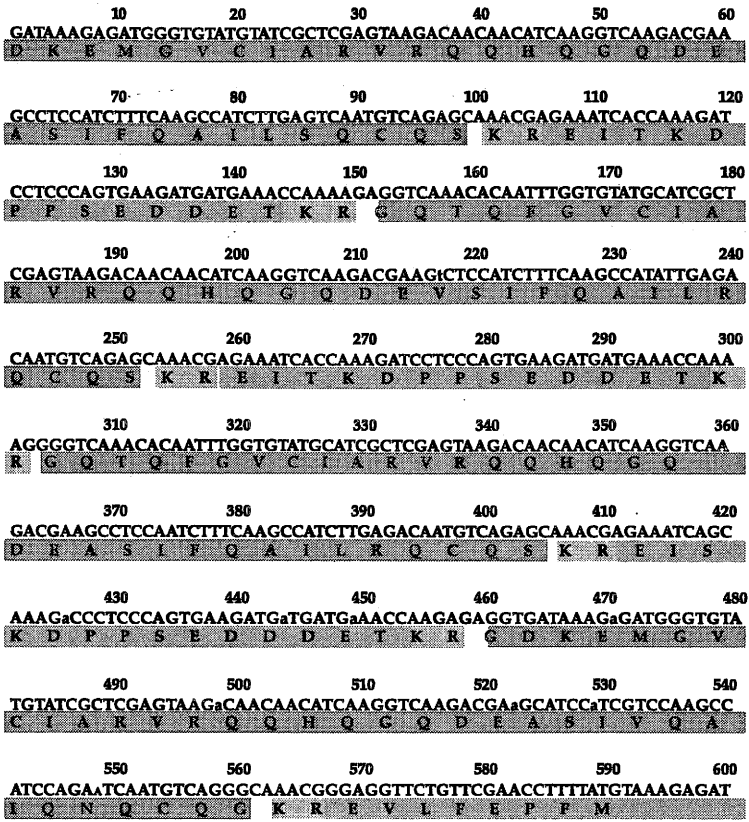


Figure 7. Partial nucleotide and amino acid sequences of 1.8 kb insert (SAP 4). The numbers located on top are the nucleotide positions. Dark shaded amino acids represent the asterosaps and the pro-peptide fragments are shaded with gray. Lys-arg residues are shaded with light gray.

Gly-Gln-Asp-Glu-Ala-Ser-Ile-Phe-Gln-Ala-Ile-Leu-Arg-Gln-Cys-Gln-Ser) corresponding to asterosap 13-2 (Figure 1) occurred twice. The amino acid sequence also showed a third asterosap with peptide structure of Gly-Ala-Ser-Gln-Phe-Gly-Leu-Cys-Ile-Ala-Arg-Val-Arg-Gln-Lys-His-Gln-Gly-Gln-Asp-Glu-Ala-Ser-Ile-Phe-Gln-Ala-Ile-Leu-Ser-Gln-Cys-Gln-Ser which corresponds to asterosap 13-1 (Figure 1).

SAP 4 also showed the presence of asterosaps 9-1 and 13-2 (Figure 1). However, two isoforms (4A & 4B) with some variations in the sequence were observed. 4A had an encoding analogue of Gly-Gln-Thr-Gln-Phe-Gly-Val-Cys-Ile-Ala-Arg-Val-Arg-Gln-Gln-His-Gln-Gly-Gln-Asp-Glu-Val-Ser-Ile-Phe-Gln-Ala-Ile-Leu-Arg-Gln-Cys-Gln-Ser similar to asterosap 13-2 with a variation at position 22 from the amino terminal. Instead of alanine, valine was observed. The predicted peptide structure for asterosap 4B (Gly-Gln-Thr-Gln-Phe-Gly-

Val-Cys-Ile-Ala-Arg-Val-Arg-Gln-Gln-His-Gln-Gly-Gln-Asp-Gln-Ala-Ser-Ile-Val-Gln-Ala-Ile-Gln-Asp-Gln-Cys-Gln-Gly) is similar to asterosap P3 (Fig. 1) but with variation at position 25 from the amino terminal. Phenylalanine was replaced with valine.

Subcloning of 2.2 and 1.8 kb inserts in Sma 1 and Stu 1 generated a single DNA molecule (Figure 8). Deduced amino acid sequence predicted another two new isoforms of asterosaps which were not found in egg jelly coat. Isoform 4C is similar to 13-1 but with substitution at position 25 from the amino terminal with valine instead of phenylalanine while isoform 4D is similar to 13-2 with substitution also observed at position 25 from the amino terminal with valine instead of phenylalanine.

The deduced amino acid sequence of the pro-peptide fragment predicted a structure of dibasic amino acid pair of Lys-Arg in both N and C termini.

```

10      20      30      40      50      60
CACGAGGATAAAGAGATGGGTGTATCGCTCGAGTAAGACAACACATCAAGGTC
H E D K E M C V C I A R V R Q Q H Q G Q

70      80      90      100     110     120
AAGACGAAGCCTCCATCTTTCAAGCCATCTTGAGTCAATGTCAGAGCAAACGAGAAATCA
D E A S I F Q A I L S Q C Q S K R E I T

130     140     150     160     170     180
CCAAAGATCTCCCACTGAAGATGATGAAACCAAAAGAGGTCAAACACAATTTGGTGTAT
K D P P S E D D E T K R G Q T Q F G V C

190     200     210     220     230     240
GCATCGCTCGAGTAAGACAACACATCAAGGTCAAGACGAAGTCTCCATCTTTCAAGCCA
I A R V R Q Q H Q G Q D E V S I F Q A I

250     260     270     280     290     300
TAITGAGACAATGTCAGAGCAAACGAGAAATCAACAAAGATCTCCCACTGAAGATGATG
L R Q C Q S K R E I T K D P P S E D D E

310     320     330     340     350     360
AAACCAAAAGAGGTCAAACACAATTTGGTGTATCGCTCGAGTAAGACAACACATC
T K R G Q T Q F G V C I A R V R Q Q H Q

370     380     390     400     410     420
AAGGTCAAGACGAAGCCTCCATCTTTCAAGCCATCTTGAGACAATGTCAGAGCAAACGAG
G Q D E A S I F Q A I L R Q C Q S K R E

430     440     450     460     470     480
AAATCAGCAAGACCTCCCACTGAAGATGATGATGAAACCAAGAGAGGTGATAAGAGA
I S K D P P S E D D E T K R G D K E M

490     500     510     520     530     540
TGGGTGTATGTCGCTCGAGTAAGACAACACATCAAGGTCAAGACGAAGCCTCCATCT
G V C I A R V R Q Q H Q G Q D E A S I F

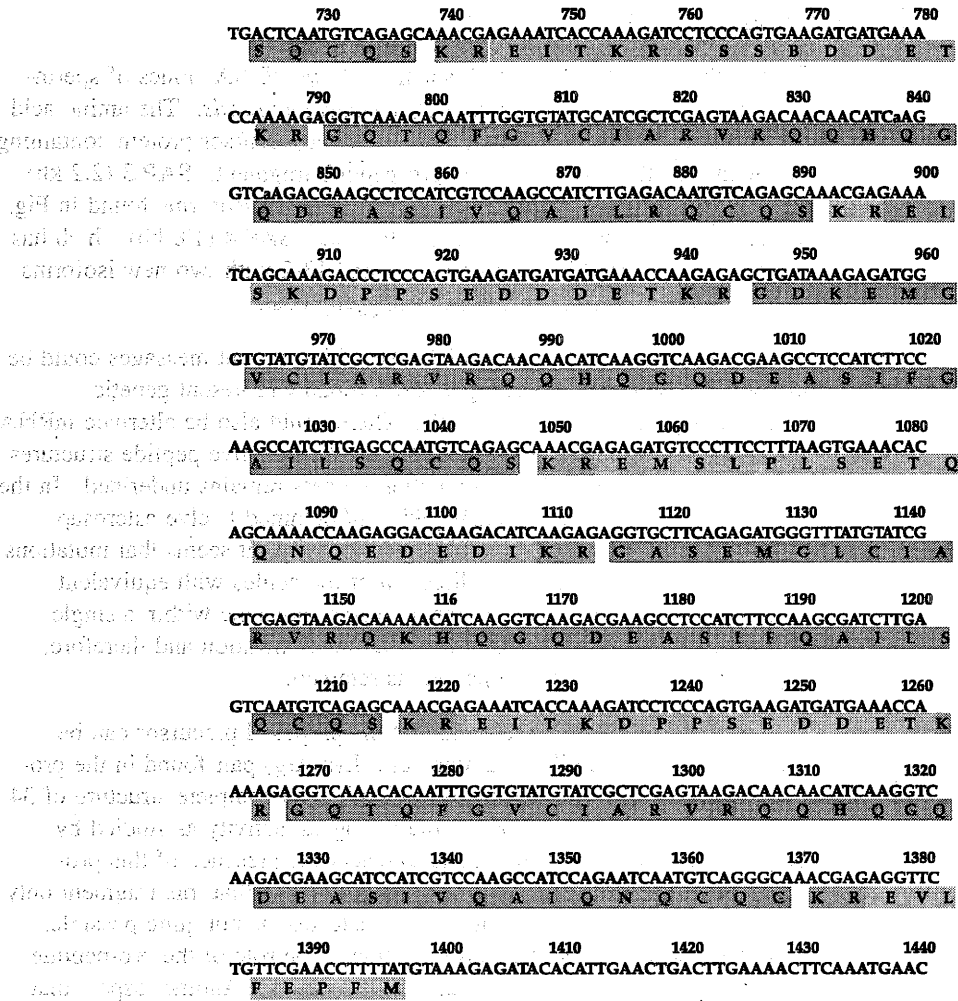
550     560     570     580     590     600
TCCAAGCCATCTTGAGCCAATGTCAGAGCAAACGAGAGATGTCCTTCCTTTAACTGAAA
Q A I L S Q C Q S K R E M S L P L S E T

610     620     630     640     650     660
CACAGCAAAACCAAGAGGACGAAGACATCAAGAGAGGTGCTTCAGAGATGGGTTTATGTA
Q Q N Q E D E D I K R G A S E M G L C I

670     680     690     700     710     720
TCGCTCGAGTAAGACAACCAACATCAAGGTCAAGACGAAGCCTCCATCGTCCAAGCCATCT
A R V R Q K H Q G Q D E A S I V Q A I L

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SAP 3



SAP 3

Figure 8. Partial nucleotide and amino acid sequences of 2.2 kb and 1.8 kb inserts (SAP 3 and 4). The numbers located on top are the nucleotide positions. Dark shaded amino acids represent the asterosap and the pro-peptide fragments are shaded light gray.

DISCUSSION

This study showed a successful isolation of two cDNA clones of sperm-activating peptides from an ovary of *Asterias amurensis*. The amino acid sequence deduced from the clones predicted a precursor protein containing alternating units of asterosap and pro-peptide fragment. SAP 3 (2.2 kb) contains three asterosaps similar to 9-1, 13-2, 13-1 isoforms found in Fig. 1 which were isolated from the egg jelly coat. SAP 4 (1.8 kb) which has a shorter length contains asterosaps 9-1 and 13-2 with two new isoforms which have not been detected in the egg jelly coat.

The existence of the 2.2 kb and 1.8 kb with different messages could be the product of two non allelic genes or probably represent genetic variation among starfish individuals. There could also be alternate mRNA splicing that occurs. The need to retain multiple active peptide structures within a single mRNA during evolution process remains undefined. In the case of *Asterias amurensis*, Nishigaki *et al* obtained twelve asterosap isoforms with almost the same biological activity. It seems that mutations of asterosaps tend to be in the direction of molecules with equivalent activity. This could mean that one asterosap molecule within a single mRNA would not be sufficient for effective fertilization and therefore, multiple copies of the active peptide is retained.

Another alternative explanation is that the proposed precursor can be cleaved between the dibasic amino acid (Lys-Arg) pair found in the pro-peptide fragment. The asterosaps which have a complete structure of 34 amino acid residues only possess the biological activity as studied by Nishigaki *et al*. They were unable to detect the presence of this pro-peptide fragment in the egg jelly coat. It could be that this fragment only exists in a very low concentration, thus detection is not quite possible. But one aspect that needs an investigation is the role of the pro-peptide fragment with the asterosaps in acrosome reaction. Another aspect that also needs consideration is when or where the precursor polypeptide is formed. One significant phase of this study is the availability of cDNA that can express the precursor in cells that do not normally produce sperm activating peptides and once an active molecule is released, then site directed mutagenesis may reveal the importance of asterosaps and pro-peptide fragment within the same polypeptide.

An interesting feature of this asterosap precursor molecule is the separation of asterosap from the pro-peptide fragment by cleavage at the dibasic amino acid pair of Lys-Arg residues. This can be carried out if a trypsin-like protease is present. Then the precursor could be processed by carboxypeptidase B activity that specifically hydrolyzes the carboxyl side

of lysine and arginine residues. Trypsin-like proteases were found to be present in eggs according to Sawada et al, 1984 and also found in cortical granules of *S. purpuratus* and other species of sea urchins (Alliegro & Schuel, 1985 & 1988). Many neuropeptides and hormones are initially synthesized as large precursor molecules.

SUMMARY

1. An ovary of *Asterias amurensis* contains mRNA of sperm activating peptides (SAPs).
2. The mRNA of SAP is largely distributed in oocytes than in follicle cells.
3. Two specific clones (SAP 3, 2.2 kb and SAP 4, 1.8 kb) from cDNA library were obtained.
4. Partial DNA sequence predicted the structure of SAP 3 to contain asterosaps 9-1, 13-2 and 13-1, the same asterosaps isolated from egg jelly coat which has the capability of activating homologous spermatozoa and inducing acrosome reaction. The deduced amino acid sequence predicted the presence of a pro-peptide fragment which is made up of dibasic amino acid pair of Lys-Arg in both N and C termini.
5. SAP 4 was predicted to contain asterosap 9-1 and 13-2 with two new isoforms of asterosaps that were not observed in the egg jelly coat.
6. Deduced amino acid sequence of the two new isoforms 4A and 4B predicted a peptide structure of Gly-Gln-Thr-Gln-Phe in the N-terminal region. Isoform 4A is similar to asterosap 13-2 but with a substitution of alanine with valine at position 22 from the amino terminal. Isoform 4B has the same amino acid sequence with asterosap P3. Variation was observed at position 25 from the amino terminal with the presence of valine instead of phenylalanine.
7. Subcloning of 2.2 kb and 1.8 kb inserts in Sma 1 and Stu 1 generated a single DNA molecule. Deduced amino acid sequence predicted another two new isoforms of asterosaps which were not found in egg jelly coat. Isoform 4C is similar to 13-1 but with substitution at position 25 from the amino terminal with valine while isoform 4D is similar to 13-2 with substitution also observed at position 25 from the amino terminal with valine.

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Comparative Antidiabetic Activities of Some Medicinal Plants

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ABSTRACT

The methanolic and aqueous extracts from popular, abundant and relatively cheap sources such as banana flowers, pipino fruits, and leaves of ampalaya, kangkong, kamote, makopa, and tanglad were tested for their comparative antidiabetic activities using albino mice as test animals. The blood glucose levels were determined using haemoglucotest strips.

The results of the bioassay of the aqueous extracts showed that the most potent antidiabetic plant is banana flowers while the comparative antidiabetic potencies of the other plants are: tanglad leaves > pipino fruits > kangkong leaves > makopa leaves > ampalaya leaves > kamote leaves. An infusion from mango leaves did not exhibit antidiabetic activity. The organic extract of makopa leaves is the most antidiabetic followed by kangkong and mango leaves. The organic extracts of banana flowers and kamote, ampalaya, and tanglad leaves exhibited low antidiabetic potentials.

INTRODUCTION

Diabetes is one of the top ten diseases in the Philippines. Diabetes mellitus is a disorder of glucose metabolism that results from the inadequate secretion of insulin from the pancreas. Insulin, tolbutamide, chlorpropamide, acetohexamide, tolazamide, glipizide and glyburide are drugs commonly used to control the blood glucose levels. However, all these hypoglycemic drugs are expensive.

Several antidiabetic Philippine medicinal plants are listed in the Medicinal Plants of the Philippines (Quisumbing, 1978). This research is on the dose-response relationships of the organic and aqueous extracts from banana (*Musa sapientum*) flowers, pipino (*Cucumis sativus*) fruits, and leaves of ampalaya (*Momordica charantia*), kangkong (*Ipomoea aquatica*), kamote (*Ipomoea batatas*), makopa (*Syzygium malaccense*), mango (*Mangifera indica*) and tanglad (*Andropogon citratus*) using albino mice as test animals. A fast screening bioassay using haemoglucotest strips was done. The results of this study will establish the relative antidiabetic potencies of these medicinal plants.

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MATERIALS AND METHODS

Preparation of Plant Samples

Fresh banana flowers, pipino fruits, and leaves of ampalaya, kangkong, kamote, tanglad, mango, and makopa were bought from Fairview wet market. Expression of one pipino fruit weighing 142.4 grams was done by using a homogenizer. Filtration of the homogenate yielded 110 ml of the filtrate. Infusions from banana flowers, and leaves of ampalaya, kangkong, kamote, tanglad, mango, and makopa were prepared by separately soaking 20g in enough distilled water for 30 minutes at room temperature. The mixture was then filtered and distilled water was added to make a final volume of 15.5 ml.

Air-dried plant samples were soaked in enough methanol for several days. Concentrated crude methanol extracts were obtained using a rotavapor (Heidolph). Watery methanol extracts from kamote and kangkong leaves were partitioned with ethyl acetate and the ethyl acetate layers were subsequently concentrated *in vacuo*.

Antidiabetic Bioassay

Three albino Swiss Webster mice were used per test sample. The mice were fasted for 12 hours with constant supply of water. They were then individually weighed.

The blood glucose levels of three mice were determined to know their normal glucose levels (time = 0 min.) using the haemoglucotest strips (Haemo-Glukotest R by Boehringer Mannheim). Blood was extracted by the tail venipuncture method. The second drop of blood was placed on the haemoglucotest strip to completely cover the reactive site of the strip. After 60 seconds, the blood was wiped immediately applying light pressure using a clean cotton ball. The results were obtained after 2 minutes by comparing the color of the test site with the color of the block chart provided in the container of the haemoglucotest strips. The corresponding reading in mmol/L was then recorded.

An increase in blood glucose levels (hyperglycemia) of the mice was immediately induced by injecting 0.20 ml of 75% of sugar solution per 20 g mouse. Using the same procedure as above, the corresponding glucose concentration in mmol/L was recorded using 30 minutes interval. This is the positive control.

The mice were injected intraperitoneally with 0.2 ml of the plant infusion per 20 g mouse immediately upon onset of hyperglycemia. Using the same procedure as above, the corresponding glucose concentration in mmol/L was recorded using 30 minutes intervals.

The concentration of the plant infusions is 1.29 g / ml based on pipino fruit. The dosage for the organic extracts is 10 mg per 20 g mouse using dimethylsulfoxide (DMSO) or 50% DMSO as solvent.

RESULTS AND DISCUSSION

The pipino fruit has the highest water content among the seven samples. A fruit weighing 142.4 g gave a total infusion volume of 110 ml for a concentration of 1.29 g / ml. Hence, 20 g of the other plants were separately soaked in enough distilled water for 30 min to make a final volume of 15.5 ml

For the control group whose normal blood glucose level is 4 mmol/L, injection of 75% sugar solution led to an increase in blood glucose level to 7.7 mmol/L after 30 minutes, 9 mmol/L after 60 minutes, and 6 mmol/L after 90 minutes. After 90 minutes, although the blood glucose level has decreased, it has not yet reached its normal level.

Table 1 and Figure 1 shows that the most effective antidiabetic plants are banana flowers and tanglad leaves. The blood glucose levels for banana flowers and tanglad leaves decreased by 57.1% and 46%, respectively, 30 minutes after injection of the plant infusions. Also, the blood glucose levels revert back to the normal levels after this time interval.

Table 1. Comparative blood glucose levels (in mmol/L) of mice injected with Aqueous extracts of plant samples.

Aqueous Extracts from Plant Samples	Normal Glucose Levels (mmol/L) ¹ (time=0 min.)	Blood Glucose Levels ² Prior to Injection of Plant Samples	Blood Glucose Levels After Injection of Plant Samples		
			30 min	60 min	90 min
Control Group	4.0	6.0	7.7	9.0	6.0
Kamote leaves	3.3	6.0	6	7.5	9 ³
Mango leaves	2.0	6.0	10.0	8.0	
Pipino leaves	3.0	7.0	3.3	2 ³	1 ³
Ampalaya leaves	4.0	7.7	7	5.7	4 ³
Kangkong leaves	4.0	7.7	5	4 ³	
Banana flowers	4.0	7.7	3.3		
Makopa leaves	2.7	5.0	3.3	2 ³	
Tanglad leaves	2.7	5.0	2.7		

¹prior injection of 0.2 ml of 75% sugar solution per 20 g mouse

²upon onset of hyperglycemia; average of blood glucose levels of three mice

³blood glucose level of one mouse

Antidiabetic Potencies of Aqueous Extracts

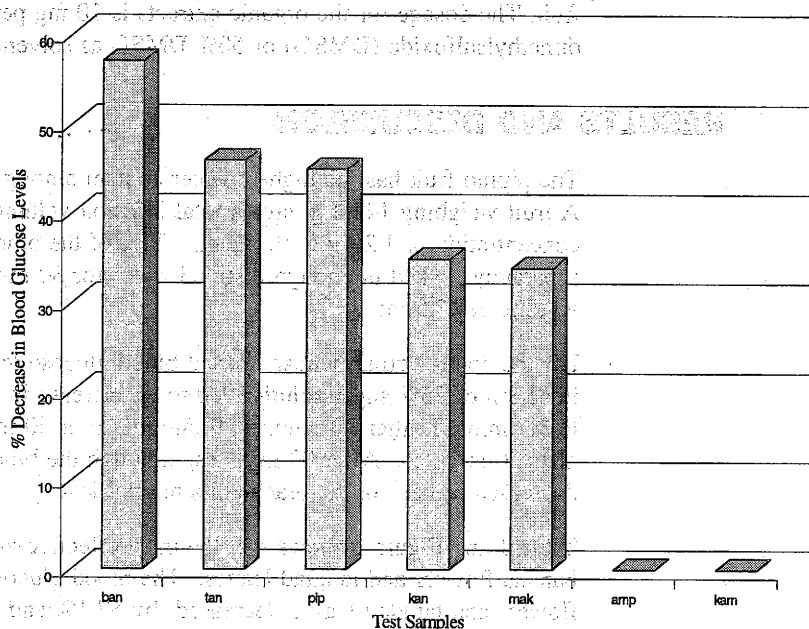


Figure 1. Comparative anti-diabetic potencies thirty minutes after injection of aqueous extracts.

There was also a 45%, 35%, and 34% decrease in the blood glucose levels of the pipino, kangkong, and makopa groups, respectively, 30 minutes after treatment. In all cases, it took 60 minutes for 1 mouse in each group to normalize its blood glucose levels.

During the same 30 minute time interval, there was no decrease in the blood glucose levels of mice injected with kamote and ampalaya leaves. For the kamote and ampalaya groups, after 60 minutes, the blood glucose levels of two out of three mice per group normalized but 1 mouse per group even showed an increase in their blood glucose levels. It took 90 minutes for the blood glucose level of the mouse in the ampalaya group and 120 minutes for the kamote group to normalize.

An infusion from mango leaves showed an increase in blood glucose levels.

Table 2 and Figure 2 showed that the organic extracts from makopa, kangkong, and mango leaves decreased blood glucose levels by 46.7%, 33.3%, and 21.4%, respectively, after a 30 min interval. Bioassays of the leaves of ampalaya, kamote, and tanglad, and banana flowers showed an increase in blood glucose levels 30 min after injection of plant samples by 66.7%, 56.2%, 27.3%, and 7.14%, respectively.

Table 2. Comparative blood glucose levels (in mmol/L) of mice injected with Aqueous extracts of plant samples.

Organic Extracts from Plant Samples	Normal Glucose Levels (mmol/L) ¹ (time=0 min.)	Blood Glucose Levels ² Prior to Injection of Plant Samples	Blood Glucose Levels After Injection of Plant Samples		
			30 min	60 min	90 min
Control Group	3		5.0	6.5	8.5
Kamote leaves	5.5	11.0	14.0	22.5	13.0
Mango leaves	4	7.0	5.5	7.0 ³	
Ampalaya leaves	3.3	7.7	9.0	13.0	9.0
Kangkong leaves	3.3	6.0	4.0	4.0 ³	
Banana flowers	3.0	7.0	7.5	9.0 ³	5.0 ³
Makopa leaves	3.0	7.5	4.0		
Tanglad leaves	4.0	8.0	12.5	18.0	19.5

¹prior injection of 0.2 ml of 75% sugar solution per 20 g mouse
²upon onset of hyperglycemia; average of blood glucose levels of three mice
³blood glucose level of one mouse

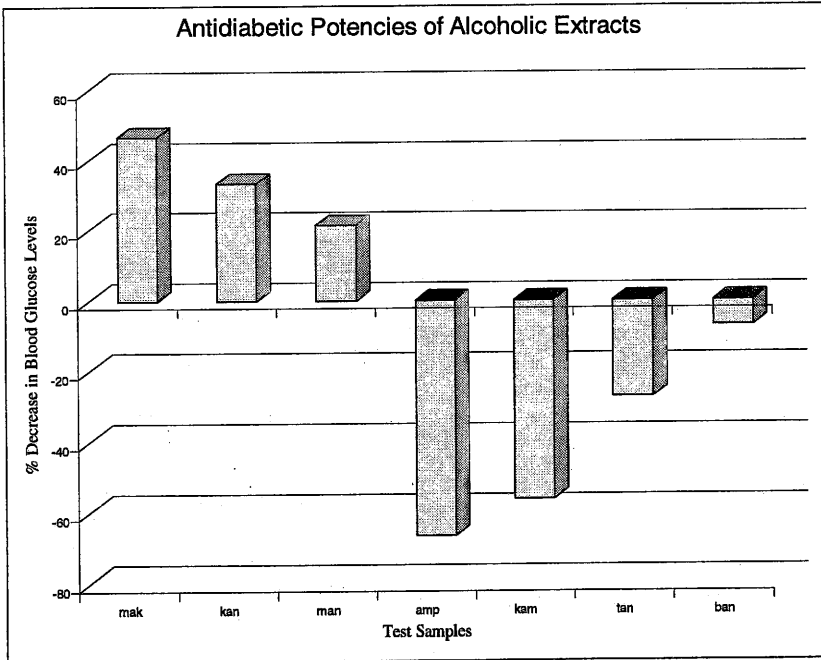


Figure 2. Comparative anti-diabetic potencies thirty minutes after injection of methanolic extracts.

CONCLUSION

Based on above results, it can be concluded that, among the aqueous extracts of the medicinal plants bioassayed, the most potent antidiabetic plant is banana flowers. The comparative antidiabetic potencies of the other plants are: tanglad > pipino > kangkong > makopa > ampalaya > kamote. An infusion from mango leaves did not exhibit antidiabetic activity.

The relative antidiabetic potencies of the organic extracts of the medicinal plants at a dosage of 10 mg per 20 g mouse are: makopa leaves > kangkong leaves > mango leaves. Extracts of kamote, ampalaya, and tanglad leaves did not exhibit antidiabetic activities 30 minutes after intraperitoneal injection.

These results showed that the antidiabetic constituents of common vegetables are present in the aqueous extracts while those of makopa and mango leaves are present in the organic or alcoholic extracts. This study support the method of administration of these common vegetables as food, expression, or tea.

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A Bioactive Dipeptide Derivative from *Malachra fasciata*

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ABSTRACT

The chloroform extract of the air-dried leaves of *Malachra fasciata* afforded aurantiamide acetate (**1**). The structure of **1** was elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by synthesis. Aurantiamide acetate was found to be active against *P. aeruginosa*, *B. subtilis*, *C. albicans* and *T. mentagrophytes* and inactive against *S. aureus*, *E. coli* and *A. niger* at a dosage of 80 µg. It was found to reduce the number of micronucleated polychromatic erythrocytes induced by mitomycin C by 32%.

Keywords: *Malachra fasciata*, Malvaceae, aurantiamide acetate, lyciumamide, asperglaucide, antimicrobial, antimutagen

INTRODUCTION

Malachra fasciata is a shrub found throughout the Philippines at low altitudes. Poultices of the leaves are applied to ulcers and other sores. The roots and leaves may serve as emollients and may be considered specific against haemorrhoids, fevers and impotency and also as a general tonic. A decoction of the leaves is used for treatment of gonorrhoea and rheumatism and as a demulcent and diuretic [Quisumbing, 1978]. An earlier study on *Malachra fasciata* reported the isolation, structure elucidation and antimutagenic property of loliolide from the chloroform extract of the leaves of the plant [Ragasa *et.al.*, 1997].

We now report the isolation, structure elucidation, antimicrobial and antimutagenic activities of aurantiamide acetate (**1**). This is the first report on the isolation of **1** from *M. fasciata*, its structure elucidation by NMR spectroscopy, and its antimicrobial and antimutagenic properties. The structure of **1** was confirmed by synthesis. Compound **1** was previously isolated from *Piper auranticum* [Benerji, *et.al.*, 1981], *Arisaema erubescens* [Ducki, *et.al.*, 1996], *Aspergillus glaucus* [Cox, *et.al.*, 1996] and *Cystoseira corniculata* [Maiti, *et.al.*, 1976].

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RESULTS AND DISCUSSION

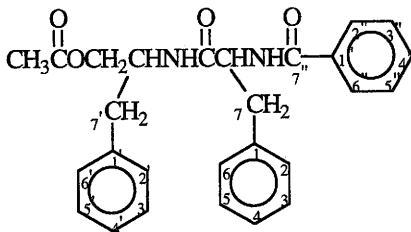
The ^1H NMR spectral data of **1** (Table 1) indicated resonances for an acetate methyl at δ 2.0 (3H, s), benzylic methylene protons at δ 2.73 (1H, dd, $J = 7.5, 13.7$ Hz), 2.76 (1H, dd, $J = 6.9, 13.7$ Hz), and δ 3.05 (1H, dd, $J = 8.5, 13.7$ Hz), 3.23 (1H, dd, $J = 6.0, 13.7$ Hz), methylene carbonyl

Table 1. 500 MHz ^1H and 125 MHz ^{13}C NMR spectral data of **1.**

Carbon	^{13}C , δ	^1H , δ
C-1	136.6	---
C-2, C-6	129.3	7.24
C-3, C-5	128.8 ^a	7.27
C-4	127.2	7.21
C-7	38.4	3.05, 3.23
C-8	55.0	4.76
C-9	170.2	---
C-1'	136.7	---
C-2', C-6'	129.2	7.07
C-3', C-5'	128.7 ^a	7.15
C-4'	126.8	7.12
C-7'	37.5	2.73, 2.76
C-8'	49.5	4.35
C-9'	64.6	3.80, 3.93
OBenz		
C-1"	133.7	---
C-2", C-6"	127.1	7.70
C-3", C-5"	128.6 ^a	7.42
C-4"	132.0	7.51
C-7"	167.1	---
OAc		
CH_3CO	170.8	---
CH_3CO	20.7	2.03
---	---	5.90 (NH)
---	---	6.75 (NH)

^a Assignments may be interchanged.

protons at δ 3.93 (1H, dd, $J = 4.7, 11.4$ Hz) and 3.80 (1H, dd, $J = 4.4, 11.4$ Hz), methine carbonyl protons at δ 4.35 (1H, m) and 4.76 (1H, m), protons attached to N at δ 5.90 (d) and 6.75 (d), aromatic protons at δ 7.07 (2H, m), 7.12 (1H, m), 7.15 (2H, m), 7.21 (1H, m), 7.24 (2H, m), 7.27 (2H, m) and 7.42 (2H, m), 7.51 (1H, m) and 7.70 (2H, m).



1

The COSY spectrum indicated the following four isolated spin systems. The methylene protons at δ 3.99 and 3.80 are coupled to the methine hydrogen at δ 4.35, which is in turn coupled to the benzylic protons at δ 2.74, 2.76 and the hydrogen attached to a nitrogen at δ 5.90; the benzylic methylene protons at δ 3.05 and 3.23 are coupled to the methine hydrogen at δ 4.76, which is in turn coupled to the amide proton at δ 6.76; the aromatic protons at δ 7.42 (2H) are coupled to the hydrogens at δ 7.70 (2H), which are in turn coupled to the proton at δ 7.51 (1H). The remaining aromatic protons form two complex spin systems comprising the protons at δ 7.07 (2H), 7.12 (1H) and 7.15 (2H), and at 7.21 (1H), 7.24 (2H) and 7.27 (2H). Thus, the molecule contains three mono-substituted benzene rings.

The ^{13}C NMR spectral data (Table 1) showed resonances for the carbonyl of an ester at δ 173, the carbonyls of amides at δ 170.2 and 167.1, the carbonyl carbon at δ 64.6, protonated aromatic carbons at δ 127.1-132.0 and non-protonated aromatic carbons at δ 136.6, 136.7 and 133.7, nine protonated aromatic carbon resonances (six representing two carbons each) between δ 127.1-129.3, the carbonyl carbon at δ 64.6, two carbons attached to nitrogen at δ 55.0 and 49.5, two benzylic methylene carbons at δ 38.4 and 37.5, and an acetate methyl carbon at δ 20.7.

The ^{13}C and ^1H assignments for **1** were verified by a short-range heteronuclear 2D experiment HMQC (Table 1) and connectivity was verified by the inverse long-range heteronuclear experiment HMBC optimized for $J = 10$ Hz (Table 2). All long-range correlations observed were consistent with the proposed structure of **1**.

Table 2. 300 MHz HMBC spectral data of 1.

Carbon No.	^1H , δ
C-1	H-3, H-5, H-7
C-2, C-6	H-4, H-7
C-3, C-5	---
C-4	H-2, H-6
C-7	H-2, H-6, H-8
C-8	H-7
C-9	H-7, H-8

C-1'	H-7'
C-2', C-6'	H-4', H-7'
C-3', C-5'	---
C-4'	H-2', H-6'
C-7'	H-2', H-6'
C-8'	H-7'
C-9'	H-7'
OBenz	
C-1"	H-3", H-5"
C-2", C-6"	H-4"
C-3", C-5"	
C-4"	H-2", H-6"
C-7"	H-2", H-6"
OAc	
C=O	2.0-CH ₃
CH ₃	

The relative stereochemistry of **1** was determined by NOESY which indicated correlation through space of the ^1H nuclei in the molecule (Table 3).

Literature search revealed that **1** is aurantiamide acetate (also known as lyciumamide and asperglaucide). The structure and stereochemistry of **1** has been previously confirmed by synthesis [Ducki, et.al., 1996].

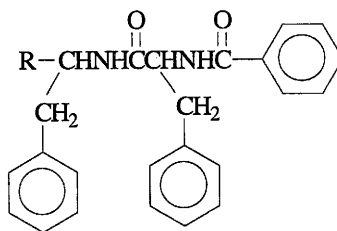
Because limited amounts of **1** were isolated, a synthesis was carried out to afford more sample for antimicrobial and antimutagenicity tests. Di-L-

Table 3. 300 MHz NOESY correlation of 1.

Proton No.	¹ H,—Correlation
H-9'	H-8'
H-8'	H-9', H-7'
H-7'	H-8', 5.90-NH
H-2', H-6'	H-3', H-5'
H-3', H-5'	H-2', H-6', H-4'
H-4'	H-3', H-5'
H-8	H-7', 5.90-NH
H-7	H-8
H-2, H-6	H-3, H-5
H-3, H-5	H-2, H-6, H-4
H-4	H-3, H-5
H-2'', H-6''	H-3'', H-5''
H-3'', H-5''	H-2'', H-6'', H-4''
H-4''	H-3'', H-5''

phenylalanine (2) was benzoylated with benzoyl chloride to give the amide 3, which was reduced with sodium borohydride via an intermediate mixed anhydride to the alcohol 4. The alcohol was then acetylated with acetic anhydride in pyridine to yield 1. The ¹H NMR spectrum of synthetic material was identical to that of the natural product.

Compound 1 was tested for its antimicrobial properties by the agar cup method. Seven microorganisms used in the tests are as follows:



- 2 R = COOH
 3 R = CH₂OH
 4 R = CH₂OAc

Staphylococcus aureus and *Bacillus subtilis* (gram positive bacteria); *Escherichia coli* and *Pseudomonas aeruginosa* (gram negative bacteria); *Candida albicans* (yeast-like fungi); *Aspergillus niger* (multicellular mold); and *Trichophyton mentagrophytes* (parasitic fungi). Results of the study (Table 4) indicated that 1 was active against *P. aeruginosa*, *B. subtilis*, *C.*

Table 4. Antimicrobial test results on 1 and 3.

Sample	Concn. (μ g)	<i>Staphylococcus aureus</i>		<i>Escherichia coli</i>		<i>Pseudomonas aurugenosa</i>		<i>Bacillus subtilis</i>		<i>Candida albicans</i>		<i>Aspergillus niger</i>		<i>Trichophyton mentagrophytes</i>	
	C.Z (mm)	C.Z (mm)	A.I (mm)	C.Z (mm)	A.I (mm)	C.Z (mm)	A.I (mm)	C.Z (mm)	A.I	C.Z (mm)	A.I	C.Z (mm)	A.I	C.Z	A.I
1	80	-----	0	-----	0	12, 12	0	11, 13	0.2	21, 21	1.1	-----	0	14, 11	0.25
2	80	-----	0	-----	0	14, 15	0	12, 14	0.3	20, 21	1.05	-----	0	16, 16	0.6
Standard Antibiotic	30	35	2.5	35	2.5	17	0.7	21	1.1	25	1.5	31	2.1	40	3.0
		Chloramphenicol		Tetracycline		Tetracycline		Chloramphenicol		Clotrimazole		Clotrimazole		Clotrimazole	

albicans and *T. mentagrophytes* with antimicrobial indices of 0.2, 0.2, 1.1 and 0.25, respectively at a concentration of 80 µg. It was inactive against *S. aureus*, *E. coli* and *A. niger* at the same concentration. Compound **3** was also tested for antimicrobial activity and found to be active against *P. aeruginosa*, *B. subtilis*, *C. albicans* and *T. mentagrophytes* with antimicrobial indices of 0.45, 0.3, 1.05 and 0.6, respectively at a concentration of 80 µg. It was inactive against *S. aureus*, *E. coli* and *A. niger* at the same concentration. Thus, **3** has higher antimicrobial activity than **1**.

Compound **1** was tested for its antimutagenicity potential by the Micronucleus test. Results of the study (Table 5) indicated that **1** at a dosage of 8 mg/kg was found to reduce the number of micronucleated polychromatic erythrocytes (MPCE) induced by mitomycin C by 32%. Thus, **1** is an antimutagen. Statistical analysis using the T-test showed that there is a significant decrease in MPCE at $\alpha = 0.01$.

Table 5. Effects of 1 on the formation of micronucleated polychromatic erythrocytes induced by mitomycin C.

Proton No.	Ave. no. of MPCE/1000 PCE $\pm \sigma^*$	% Reduction in MPCE
1	5.67 $\pm$ 0.95	32
Control	8.33 $\pm$ 1.12	

* Average of 15 slides.

CONCLUSION

The chloroform extract of the leaves of *Malachra fasciata* afforded aurenitiamide acetate which was found to have antimicrobial and antimutagenic activities.

Experimental

General:

The identity of **1** was established by extensive 1D and 2D NMR spectroscopy and confirmed by synthesis. The 500 MHz ^1H NMR and 125 MHz ^{13}C NMR spectra were recorded on a Bruker DRX 500. The 300 MHz NMR spectra were recorded on a Bruker AM Fourier Transform 300 MHz NMR spectrometer. All NMR spectra were taken in CDCl_3 . Fractions were monitored by TLC and spots were visualized by spraying the TLC plates with vanillin/ H_2SO_4 , followed by warming.

Sample Collection:

The sample was collected from Sta. Barbara, Pangasinan in September 1996. It was identified as *Malachra fasciata* at the Philippine National Museum and a voucher specimen is kept at the Chemistry Department, De La Salle University.

Isolation:

The air-dried leaves (2.2 kg) of *M. fasciata* were extracted with CHCl_3 to afford a crude extract (59.5 g) which was treated with aqueous $\text{Pb}(\text{OAc})_2$ to precipitate the pigments [Padolina, et.al., 1974]. The treated extract (6.4 g) was fractionated by silica gel chromatography using increasing proportions of Me_2CO in CHCl_3 (10% increment) as eluents. The 30% Me_2CO in CHCl_3 fraction was rechromatographed in 20% Me_2CO in CHCl_3 to afford **1**.

Preparation of 2 [Vogel, 1957; Kokotos, 1990]. Benzoyl chloride was added to di-L-phenylalanine (500 mg) dissolved in 10% NaHCO_3 (10 mL). The mixture was shaken vigorously for 30 min, then acidified with dilute hydrochloric acid, and the benzamide (420 mg) recovered by filtration. ^1H NMR (300 MHz) δ 2.94 (1H, dd, $J = 6.7, 14$ Hz), 3.1 (3H, m), 4.78 (1H, m), 4.93 (1H, m), 6.68 (1H, bd, $J = 7$ Hz), 6.84 (1H, bd, $J = 7.5$ Hz), 7.0-7.6 (13H, m), 7.67 (2H, m).

Reduction of 2 to give 3. To the benzamide **2** (420 mg) dissolved in THF (5 mL) at -10°C was added N-methylmorpholine (110 mg), followed by ethyl chloroformate (120 mg). After 10 min, sodium borohydride (120 mg) was added. Methanol (10 mL) was then added carefully at 0°C over a period of 15 min. The solution was stirred for an additional 15 min, then neutralised with 0.5 M H_2SO_4 (2 mL). The alcohol (280 mg) was recovered by extraction with ethyl acetate (3 x 10 mL). ^1H NMR (300 MHz) δ 2.68 (1H, dd, $J = 7.4, 13.6$ Hz), 2.78 (1H, dd, $J = 7.2, 13.6$ Hz), 3.07 (1H, dd, $J = 8.6, 13.4$), 3.24 (1H, dd, $J = 6.0, 13.4$ Hz), 3.45 (2H, m), 4.09 (1H, m), 4.80 (1H, m), 6.06 (1H, bd, $J = 7.8$ Hz), 6.82 (1H, bd, $J = 7.6$ Hz), 7.0-7.6 (13H, m), 7.71 (2H, m).

Acetylation of 3 to give 4. The alcohol **3** (50 mg) was dissolved in pyridine (1 mL) and acetic anhydride (1 mL), and left overnight at room temperature. The reaction mixture was then shaken with ice (20 g) for 30 min, and the acetate recovered by extraction with ethyl acetate (3 x 10 mL). ^1H NMR (300 MHz) δ 2.03 (3H, s), 2.75 (2H, m), 3.08 (1H, dd), 3.22 (1H, dd), 4.83 (1H, dd), 3.97 (1H, dd), 4.35 (1H, m), 5.78 (1H, m), 6.94 (1H, bd), 7.72 (1H, bd), 7.05-7.20 (5H, m), 7.2-7.35 (5H, m), 7.45 (2H, m), 7.54 (1H, m), 7.72 (2H, m); ^{13}C (75 MHz) δ 170.7, 170.3, 167.1, 136.7, 136.6, 133.7, 131.9, 129.3, 129.1, 128.7, 128.6, 128.5, 127.1, 127.0, 126.7, 64.6, 55.0, 49.5, 38.4, 37.4, 20.7.

Bioassay

1. Antimicrobial Tests: The microorganisms used in these tests are *Staphylococcus aureus* UPCC 143, *Bacillus subtilis* UPCC 1, *Escherichia coli* UPCC 195, *Pseudomonas aeruginosa* UPCC 244, *Candida albicans* UPCC 2168, *Aspergillus niger* UPCC 4063 and *Trichophyton mentagrophytes* UPCC 4193.

A microbial suspension containing approximately 10^8 cells/mL was prepared for each test organism for 24-hour agar culture using 0.1% peptone water. One-tenth (0.1 mL) of the bacterial suspension was transferred into pre-poured 30 mL deep nutrient agar plate, the yeast suspension into glucose yeast peptone agar plate and the fungal suspension on potato dextrose agar plate. About 5 mL of the corresponding melted agar cooled to about 45°C was immediately poured into the plate. The plate was swirled to distribute the microbial cells evenly on the plate. After the overlay agar has solidified, three 1-cm diameter holes were bored from equidistant points using a sterile cork borer.

Two 0.2 mL portions of **1** were placed in duplicate holes per organism. A similar volume of the solvent ethanol and of the corresponding antibiotic for each test organism was placed in the remaining two wells on the plate. Plates were incubated at room temperature to prevent evaporation of liquid on the petri lid that may cause interference in distribution of organisms on the surface. Bacterial and yeast plates were read after 24 hours, while the mold plate was read after three days. Clearing zones were measured in millimeters (mm). The average for the compound was taken and the antimicrobial activity index (AI) was computed.

2. Antimutagenicity Test: The test compounds (8 mg/kg mouse) dissolved in DMSO (7.5 mL/kg mouse, solvent control) were orally administered simultaneously with mitomycin C (2.75 mg/kg mouse, positive control) to mice of the Swiss strain. For the control, only mitomycin C and DMSO were administered orally to the mice. Five mice were tested for each compound and control. The second administration was carried out after twenty-four hours. Six hours after the second administration, the mice were killed by dislocation of the neck. Blood from the bone marrow was smeared on slides (three per mouse). The slides were stained with MayGrunwald and Giemsa solutions [Schmid, 1972]. The numbers of MPCE/1000 PCE were counted by the use of a high power microscope.

ACKNOWLEDGEMENTS

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Changes in Physiological and Biochemical Parameters of Promising Mulberry (*Morus indica* L.) Under Water Stress Condition

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ABSTRACT

*Three promising mulberry (*Morus indica* L.) varieties viz. K-2, S-13 and V-1 have been evaluated for change in photosynthetic rate, carbohydrate, starch, fibre, ascorbic acid, carotenoids, protein, chlorophyll "a" and "b" and proline contents under water stress condition. The photosynthetic rate of the variety K-2 was drastically fallen from 17.39 in control to 5.21 μ mole $m^{-2} s^{-1}$ under severe water stress condition. Though, carbohydrate and starch contents were not much affected under water stress of six days but decreased drastically under severe water stress. In control, ascorbic acid content of 3.089 mg/g was found in the variety S-13 as compared to 1.828 mg/g in K-2 and 1.496 mg/g in V-1, but it was drastically reduced in the variety S-13 under water stress of only six days. Protein content was constantly reduced with water stress. Proline accumulation was found more in the variety V-1 under water stress condition. The varieties S-13 and V-1 are reported superior for physiological and biochemical parameters as well as for higher silk yield under irrigated condition, were found susceptible to water stress in the present study. It is suggested, therefore, that the varieties S-13 and V-1 can further be improved by breeders and better cultural practices for drought tolerance, so that, these promising varieties can emergence as a boon for the sericulture under water stress zones particularly for tropical countries.*

Keywords: Mulberry (*Morus indica* L.), photosynthesis, carbohydrate, starch, fibre ascorbic acid, carotenoids, protein, chlorophyll, proline content, water stress.

INTRODUCTION

Mulberry (*Morus indica* L.) is the sole food plant for silkworms (*Bombyx mori* L.). Karnataka is the major player for the country's silk production. About 70% area for mulberry cultivation is rainfed and water deficit is frequently the primary factor limiting production of quality mulberry leaf. Leaf quality plays an important role in affecting silkworm characters and silk yield (Singhal and Mala, 1997). For the present study, three promising

mulberry varieties viz. K-2, S-13 and V-1 were selected. The variety V-1 has recently been evolved by this institute which gives highest leaf yield of 71.917 kg/ha/yr (Kumar and Sarkar, 1995), as well as bioassay and chemoassay studies have confirmed its superiority over other varieties (Kumar *et al.*, 1996). Till today, the variety K-2 is commonly used for silkworm rearing. The variety S-13 has been found efficient for photosynthetic rate, low light vs dark respiration ratio, uptake and translocation of nutrients and ascorbic acid content (Chakraborti *et al.*, 1996, 1997 and Chakraborti and Singhal, 1996). The full potential of mulberry can not be exploited because of limitations on physiological and biochemical processes imposed by water stress. By altering physical and chemical composition of tissues, water deficit stress modifies various aspects of plant quality (Slavik, 1974, Turner, 1979). In mulberry, water stress inhibits synthesis of carbohydrate, protein and accelerate chlorophyll decomposition, by which silkworms are adversely affected and ultimately silk yield is reduced (Singhal and Mala, 1997). Therefore, present investigation was carried out to find out the change in physiological and biochemical parameters of promising mulberry varieties under water stress condition.

MATERIALS AND METHODS

The present investigation was carried out in three promising mulberry varieties viz. K-2, S-13 and V-1 grown in pots under glass house condition maintaining room temperature of $28 \pm 2^\circ\text{C}$. All pots were filled with 10 kg of soil (soil + farm yard manure in the ratio 3:1). Five irrigation schedule of once in 3 days (T-1), 6 days (T-2), 9 days (T-3), 12 days (T-4) and 15 days (T-5) were maintained. One year old plantation in pots was pruned and after 20 days of sprouting, different irrigation schedules were started. Each pot was supplied with equal quantity of water to field capacity. For each treatment, 12 replications were maintained in randomized design. Data were collected after 70 days of pruning. Photosynthetic rate was measured by using LICOR-6200 portable photosynthesis system. Carbohydrate, starch and fibre contents were estimated by the method of AOAC (1980). Ranganna (1977) method was followed for the estimation of ascorbic acid. Protein content was estimated by using tecator auto kjeltec system. Chlorophyll and carotenoid contents were estimated by the method of Hiscox and Israelstam (1979). Bates *et al.* (1973) method was followed for proline estimation. All data were analyzed statistically.

RESULTS AND DISCUSSION

The changes in physiological and biochemical parameters in promising mulberry varieties- K-2, S-13 and V-1 under water stress are presented in Table 1 and Figures 1-10. Drastic reduction in photosynthetic rate was recorded in all the varieties under water stress (Figure 1). In the variety K-2, photosynthetic was fallen from 17.39 to 5.21 $\mu\text{mole m}^{-2}\text{s}^{-1}$ which reveals high susceptibility of K-2 to water stress. However, in the variety

Table 1. Change in physiological and biochemical parameters of some promising mulberry genotypes under water stress condition.

Treatment	Variety	Photosynthetic rate (μ mole m ⁻² s ⁻¹)	Carbohydrate content (%)	Starch content (%)	Fibre content (%)	Ascorbic acid (%)	Carotenoids (mg/g)	Protein (%)	Chl. "a" (mg/g)	Chl. "b" (mg/g)	Proline (%)
T-1	K-2	17.39	8.627	13.593	10.267	1.828	1.037	20.09	2.459	0.523	84.63
	S-13	16.24	12.990	13.673	10.067	3.089	1.025	21.71	2.316	0.857	102.43
	V-1	15.44	9.593	13.590	10.467	1.496	1.119	21.12	2.145	0.572	48.36
T-2	K-2	13.76	6.190	13.183	11.267	1.393	1.015	19.12	2.079	0.523	183.93
	S-13	13.11	12.553	12.367	16.667	1.714	0.916	18.43	2.252	0.596	142.80
	V-1	13.48	6.463	13.370	14.733	1.313	1.108	19.17	2.086	0.544	155.80
T-3	K-2	11.31	5.873	12.283	12.167	1.205	1.014	17.24	1.916	0.533	196.43
	S-13	12.04	7.567	9.800	18.567	1.711	0.857	15.26	1.618	0.588	215.83
	V-1	10.30	4.953	12.153	15.433	1.243	1.021	18.38	1.872	0.526	214.43
T-4	-2	9.52	3.353	11.060	14.267	1.011	1.021	15.13	1.734	0.385	349.00
	S-13	8.50	4.803	7.833	20.267	1.683	0.656	12.39	1.610	0.588	299.43
	V-1	8.81	4.777	11.093	16.167	1.057	0.989	15.28	1.705	0.369	394.40
T-5	-2	5.21	2.703	9.097	17.567	0.767	0.856	10.10	1.358	0.381	372.43
	S-13	5.11	3.207	6.133	21.367	1.260	0.526	9.37	1.593	0.512	372.93
	V-1	6.48	4.517	10.290	19.800	1.013	0.861	11.95	1.547	0.356	415.73
CD at 1%											
Var.		0.243	0.133	0.218	0.451	0.055	0.055	0.171	0.021	0.017	6.73
Treat.		0.314	0.172	0.281	0.582	0.071	0.071	0.221	0.026	0.023	8.68
VXT		0.544	0.298	0.487	1.009	0.123	0.123	0.38	0.046	0.039	15.04

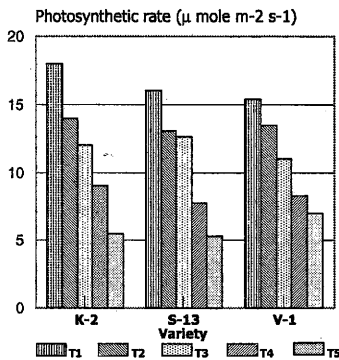


Figure 1. Photosynthetic rate

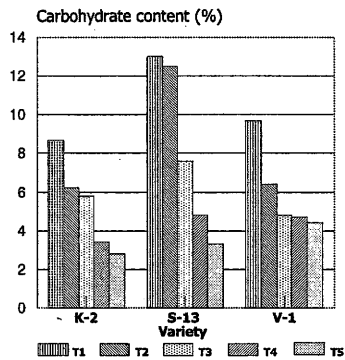


Figure 2. Carbohydrate content

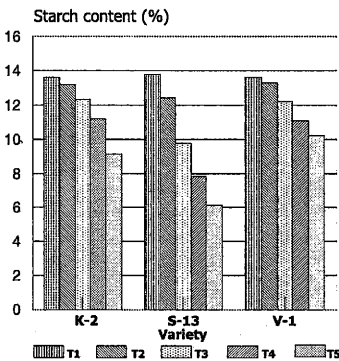


Figure 3. Starch content (%)

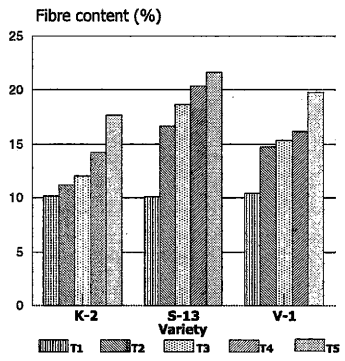


Figure 4. Fibre content (%)

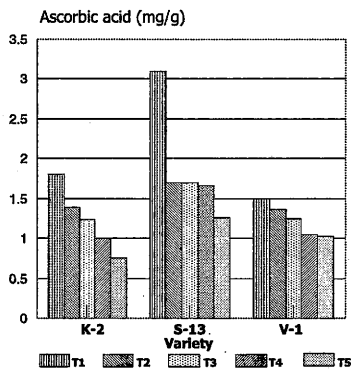


Figure 5. Ascorbic acid

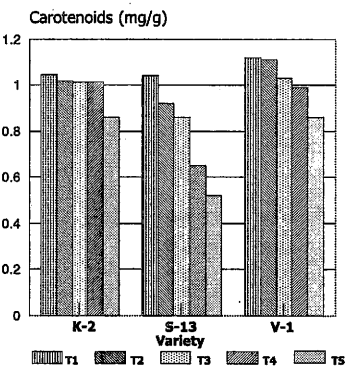


Figure 6. Carotenoids

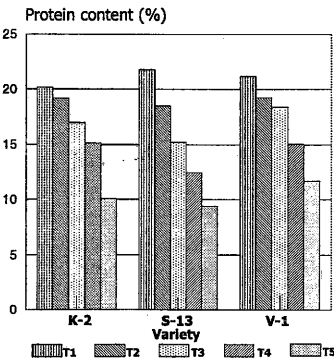


Figure 7. Protein

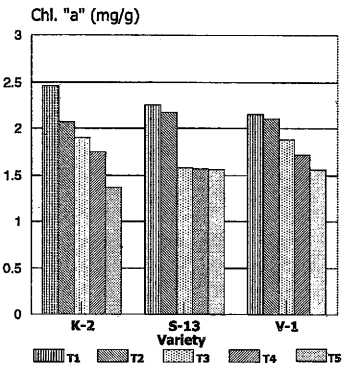


Figure 8. Chl. "a"

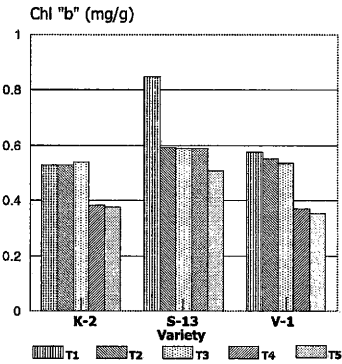


Figure 9. Chl "b"

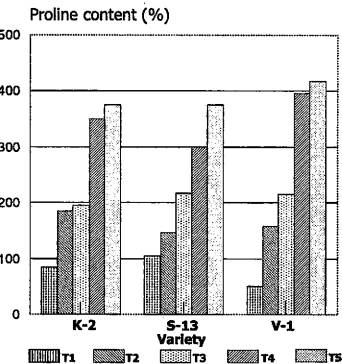


Figure 10. Proline content

V-1, fall in photosynthetic rate was comparatively slow i.e from 15.44 to 6.48 μ mole m⁻² s⁻¹. The variation found between variety, treatment and variety x treatment were found highly significant. The study also shows drastic decrease in the contents of carbohydrates (Figure 2), starch (Figure 3), ascorbic acid (Figure 5), carotenoid (Figure 6) and chlorophyll (Fig. 8&9). The variety S-13 contained highest carbohydrate, starch, ascorbic acid, protein and chlorophyll "b" contents in the treatment T-1, than all other varieties. However, under the water stress of T-5, the variety V-1 maintained comparatively higher carbohydrate (4.517%), starch (10.290%), carotenoids (0.861 mg/g) and protein content (11.95%) than all other varieties. In this regard, the variety K-2 was highly affected for carbohydrate, ascorbic acid chlorophyll "a" contents under water stress. Fibre content was increased in all the varieties with the increase in water stress (Figure 4). The protein content was almost fallen to half in the treatment T-5 in all the varieties studied (Figure 7). In the variety S-13, though carbohydrate and starch contents were not much affected up to T-2 but severely decreased under water stress of T-3 to T-5. However, this variety was found highly sensitive for ascorbic acid content which was decreased from 3.089 in T-1 to 1.714 mg/g under the water stress of T-2. Interestingly, in T-5, S-13 has significantly maintained high ascorbic acid of 1.260 mg/g than mere 0.767 mg/g in K-2

and 1.013 mg/g in V-1 varieties. Protein content was decreased comparatively at a slow rate in the variety V-1 than other varieties under water stress condition. The proline content was found increased with the intensity of water stress (Figure 10). The variety V-1 has accumulated highest proline content than other two varieties under water stress.

In tropical and sub-tropical countries practicing sericulture, water stress during mulberry plant development limits leaf production and deterioration leaf quality. This problem is usually frequent and intense under rainfed sericulture especially in regions with limited precipitation and relatively high evaporation potential. The poor leaf quality finally affects silkworm economic characters and there is a reduction in silk output (Singhal and Mala, 1997). The present investigation is an attempt to find out the change in physiological and biochemical parameters due to water stress.

Photosynthetic rate and other related gas exchange parameters are bound to decrease during any environmental stress (Chaves, 1991) and in mulberry the photosynthetic organ i.e. leaf itself is the economic yield. In the present investigation, photosynthetic rate was drastically reduced in all the varieties studied. The maximum reduction of photosynthetic rate i.e. 17.39 in T-1 to 5.21 $\mu\text{mole m}^{-2} \text{s}^{-1}$ in T-5 was recorded in the variety K-2. However, in the variety V-1 it was comparatively slow than other varieties. The effect of water stress on photosynthetic activity in other crops has been studied by Epharath *et al.* (1990), Leidi *et al.* (1993), and Ramanujam *et al.* (1997). As in the present study, the photosynthetic rate of S-13 was severely affected under water stress, it has shown photosynthetic rate of 32.87 $\mu\text{mole m}^{-2} \text{s}^{-1}$ in irrigated open fields, surprisingly, falls to mere 8.84 $\mu\text{mole m}^{-2} \text{s}^{-1}$ under rainfed condition (Chakraborti *et al.* 1996).

The growth and development of silkworms larvae and economic characters of cocoons are greatly influenced by the nutritional conditions of the mulberry leaves (Gabriel and Rapusas, 1976, Machii and Katagiri, 1991, Singhal and Mala, 1997). In the present study drastic decrease in carbohydrate, ascorbic acid, carotenoid, protein and chlorophyll contents were noted under water stress condition. The starch content, however, was not much affected up to the treatment T-2 in all the varieties, but in water stress of T-3 onwards, the variety S-13 was drastically affected for starch content. Fibre content was increased significantly with water stress, more in the variety S-13. It has been reported that high carbohydrate content is necessary for the healthy growth of the silkworms. The degree of increase in fat body glycogen and haemolymph trehalose are dependent on the carbohydrate content in food (Horie and Watanabe, 1980). However, lower amount of crude fibre in the leaves is desirable for the early stages, whereas, higher crude protein, starch and carbohydrates contents for the later stages of silkworm larvae (Anonymous, 1974, 1975). The ascorbic acid content was drastically decreased in the variety S-13 with the water

stress of only six days, however, beyond that, it was fallen slowly than other varieties. The ascorbic acid content of the variety V-1 has, however, not much decreased under water stress condition. Ito and Arai (1965), Datta Gupta *et al.* (1972), Ito (1978) have noticed that ascorbic acid is not synthesized in the silkworms and is responsible for higher silk filament length, denier values, better growth of silkworms as well as have phagostimulatory action. Hence, silkworms are totally dependent on mulberry leaf for ascorbic acid requirement. The present study suggests that in this respect the variety V-1 maintains adequate ascorbic acid under water stress condition. The protein content decreases constantly with water stress condition in all the varieties. Machii and Katagiri (1991) found direct correlation between high protein content in mulberry leaves and production efficiency of cocoon shell in silkworms. Fukuda *et al.* (1959) reported that about 70% of the silk protein produced by the silkworm is directly derived from the protein of the mulberry leaves. In this respect mulberry leaves produced under water stress condition in the present study were found to contain significantly low protein content. Comparatively, variety V-1 maintained slightly higher protein content than other varieties studied. Chlorophyll "a" was found more sensitive for the variety K-2 under water stress condition, however, variety V-1 maintains chlorophyll "a" and "b" under water stress condition than varieties K-2 and S-13. Thangaraj (1997) found chlorophyll "a" and "b" as closely associated with drought tolerance and suggested those parameters as biochemical markers in identifying drought tolerant genotypes in rice. Present study has indicated high accumulation of proline content also under water stress condition. Proline accumulation has been shown as an adaptive mechanisms of stress tolerance (Kathiresan, 1987). In the present investigation though variety S-13 has accumulated high proline content in T-1 itself but could not accumulate much under water stress condition. Contrary, the variety V-1 has shown lowest proline content in T-1 but accumulated very high proline content under water stress indicating its high adaptability towards water stress.

The present study, thus, is clearly highlighting low photosynthetic rate as well as decrease in mulberry leaf quality under water stress condition. Chahrabarti *et al.* (1997) have found out highest photosynthetic rate, low light vs. dark respiration ratio, high efficiency in uptake and translocation of nutrients as well as high ascorbic acid content in the variety S-13 (Chahrabarti and Singhal, 1996). Superior silkworm economic characters were also recorded by Raman *et al.* (1995) when silkworms were fed with the leaf of S-13. Further, highest leaf yield of V-1 variety and when fed to silkworms, very high cocoon characters and silk yield were recorded by Kumar *et al.* (1996). Based on the present study it is suggested that varieties S-13 and V-1 if improved by breeders and improved cultural practices for drought tolerance, these varieties can emerge as a boon for the sericulture under water stress zone particularly for tropical countries.

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CONCLUSION

Promising mulberry (*Morus indica* L.) varieties viz. K-2, S-13 and V-1 were evaluated for change in photosynthetic rate, carbohydrate, starch, fibre, ascorbic acid, carotenoids, protein chlorophyll "a" and "b" and proline contents under water stress condition. The varieties S-13 and V-1 are reported superior for physiological and biochemical parameters as well as for higher silk yield under irrigated condition were found susceptible to water stress in the present study. It is suggested, therefore, that the varieties S-13 and V-1 can further be improved by the breeders and better cultural practices for drought tolerance, so that, these promising varieties efficient in physiological and biochemical processes can emerge as a boon for the sericulture under water stress zones particularly for tropical countries.

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